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**A thesis submitted to Durham University in accordance with the
requirements for the degree of Doctor of Philosophy September 2013**

**Neuronal H₄ histamine receptors: role in
nociceptive pathways and pain states**

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Durham University

Supervisor: Dr Paul L. Chazot

ABSTRACT

The Histamine H₄ receptor (H₄R) is a classic G-protein coupled receptor (GPCR) and is expressed on a range of cell types in the body. To determine the roles of the H₄R in the peripheral and central nervous system, four hypotheses have been addressed. (1) The H₄R are expressed on sensory neurons in the peripheral and central nervous system; (2) The H₄R is differentially expressed during acute inflammatory and neuropathic pain states; (3) Antagonism of the H₄R with the antagonist JNJ 777120 ameliorates central inflammation observed during experimental autoimmune encephalomyelitis (EAE); (4) Antagonism of the central H₄R with JNJ 777120 has anxiolytic effects and improves memory performance in mice.

Based on immunofluorescence approaches we have demonstrated for the first time the presence of the H₄R on both putative A-delta (A δ) and C-fibre sensory neurons in the skin and dorsal root ganglia. Using the novel agonist selective H₄R agonist, VUF 8430 revealed functional expression of H₄R on primary rodent dorsal root ganglia neurons. Using quantitative immunoblotting, we provide preliminary evidence for the rodent H₄ receptor bilateral and unilateral upregulation in the skin, but not the spinal cord in the acute phase of Complete Freund's adjuvant induced inflammatory pain and nerve constriction acute neuropathic pain, respectively.

The H₄R is an attractive target for the treatment of many inflammatory disorders. However, in contrast to the beneficial anti-inflammatory effects of H₄R antagonism observed in literature, blockade of the receptor using JNJ 777120 in acute EAE (autoimmune disease) appeared to exacerbate the pathology, and increased spinal cord inflammation, demyelination, microglia activation and functional deficits. Antagonism of the central H₄R with JNJ 777120 at 10 and 20 mg/kg (i.p.) appears to affect the behaviour of Balb/c mice but there is no indication of altering anxiety or non-spatial memory performance. However, the study did provide preliminary evidence for central behavioural effects with regard to time spent in the central part of the platform. Overall, this thesis provides new evidence for multiple roles of the H₄R in the mammalian central and sensory neurons.

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Finally, I would like to thank my family and Mr Christopher Clarke who have provided continuous understanding, patience, encouragement and love over the last four years.

CANDIDATES DECLARATION

I confirm that no part of the materials presented has previously been submitted for a degree in this or any other University. If materials have been generated through joint work, my independent contribution has been clearly indicated. In all other cases, materials from the work of others has been clearly indicated, acknowledged and quotations and paragraphs indicated.

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ABBREVIATIONS

BDNF	<i>Brain derived neurotrophic factor</i>
BSA	<i>Bovine serum albumin</i>
cAMP	<i>Cyclic adenosine monophosphate</i>
CCI	<i>Chronic Constriction Injury</i>
CFA	<i>Complete Freund's Adjuvant</i>
CNS	<i>Central nervous system</i>
CREB	<i>cAMP response element binding protein</i>
DAO	<i>Diamine oxidase</i>
DMEM	<i>Dulbecco's Modified Eagle Medium</i>
DMSO	<i>Dimethyl sulphoxide</i>
DNA	<i>Deoxyribonucleic Acid</i>
DRG	<i>Dorsal root ganglia</i>
EAE	<i>Experimental Autoimmune Encephalomyelitis</i>
EDTA	<i>Ethylenediaminetetraacetic acid</i>
EGTA	<i>Ethyleneglycotetraacetic Acid</i>
ERK	<i>Extracellular signal-regulated kinase</i>
FCS	<i>Foetal Calf Serum</i>
Fluo-4	<i>Fluorescent cell permeant acetoxymethyl ester-4</i>
GPCR	<i>G-protein coupled receptor</i>
H ₁ R	<i>Histamine H1 receptor</i>
H ₂ R	<i>Histamine H2 receptor</i>
H ₃ R	<i>Histamine H3 receptor</i>
H ₄ R	<i>Histamine H4 receptor</i>
HBSS	<i>Hanks Balanced Salt solution</i>
HDC	<i>Histidine decarboxylase</i>
HNMT	<i>Histamine N-methyltransferase</i>
I.P.	<i>Intraperitoneal</i>

MAPK *Mitogen activated protein kinase*
MOG *Myelin Oligodendrocyte Glycoprotein*
mRNA *Messenger ribonucleic acid*
MS *Multiple Sclerosis*
NMDA *N-methyl-D-aspartate,*
NOR *Novel Object Recognition*
PI3K *Phosphatidylinositol-3- trisphosphate*
PLA₂ *Phospholipase A₂*
SDS *Sodium dodecyl sulphate*
TEMED *Tetramethylethylenediamine*
TMN *Tuberomammillary nucleus*

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GENERAL DISCUSSION	
1) The histamine H ₄ receptor is expressed on C-fibre sensory neurones	
2) The histamine H ₄ receptor expression is differentially effected in acute and chronic inflammatory pain conditions	
3) Antagonism of the central histamine H ₄ receptor exacerbates EAE	
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Chapter 1

Introduction

1.1 Nociceptive afferent neurons and the perception of Pain

Pain is defined as the bodily sensation which alerts the organism of a potential threat and tissue damage. The sensory component is called nociception and this is detection of noxious stimuli by unspecialised, free, unmyelinated nerve endings that transduce a variety of stimuli into nerve impulses and a protective response that generates a reflex withdrawal from the stimulus. Noxious stimuli provoke real or potential injury and cause complex behavioural strategies to avoid further contact with such stimuli (Almeida et al., 2004). While nociception refers to the neurophysiological mechanisms resulting from noxious stimuli, pain involves the perception of aversive stimuli and requires elaboration of sensory impulses.

1.1.1 Nociception

Nociception is the sensory mechanism which is used to detect noxious stimuli and the neurons that mediate nociception are termed nociceptors. Nociceptors correspond to free nerve endings and represent the distal part of a first order neuron consisting of small diameter fibres, with little myelin or unmyelinated fibres of the A δ or C-type, respectively. Under physiological conditions, pain results from specific activation of nociceptors by mechanical, chemical or thermal stimulus (Woolf and Ma, 2007) rather than through the hyperactivity of other sensory modulators. They present higher thresholds and respond progressively according to the intensity of the stimulus. On the other hand, sensitisation of nociceptors can result in reduced activation threshold responses as well as spontaneous activity (Treede et al., 1992).

1.1.2 Transduction

During the activation of peripheral nociceptors, pain transduction is conducted through myelinated A δ and unmyelinated C-fibres to the spinal cord. Signals then travel along the spinothalamic tract to the thalamus and cortex for modulation and

motor responses. Prolonged activation of nociceptors and a reduction in transduction threshold can result in chronic stimulation. Research has focused on the identification of molecules involved in order to develop analgesic treatments to treat chronic pain.

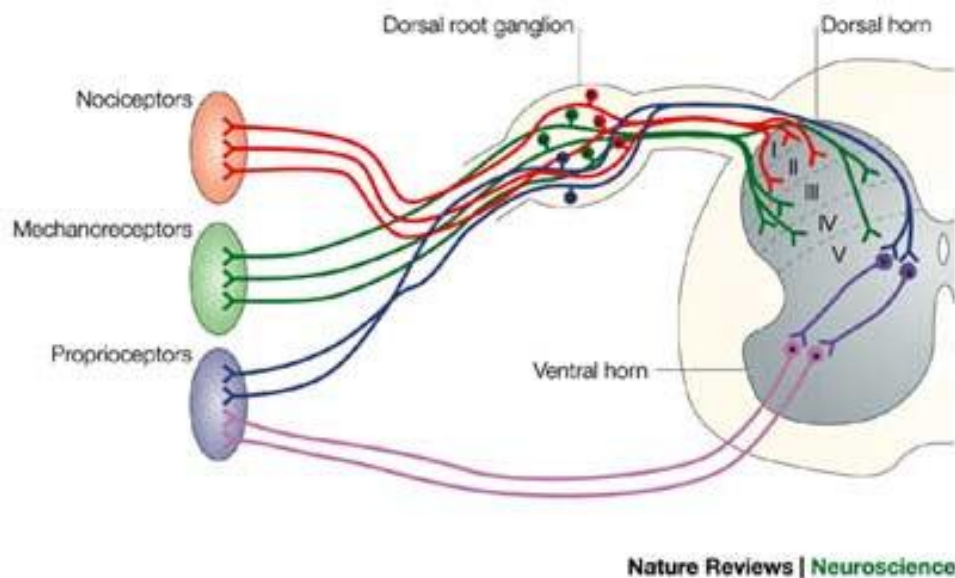


Fig 1.1 A summary of the mammalian nociceptive pathway (Caspary and Anderson, 2003)

Pain or nociception is initiated when peripheral nerve terminals (receptors) of a subgroup of sensory neurons (nociceptors) are activated by noxious chemical, mechanical or thermal stimuli. The axons of sensory neurons project to the dorsal root ganglia, where the cell bodies lie and terminate within the superficial dorsal horn of the spinal cord. Pain and temperature are detected by nociceptors and the messages are transmitted to lamina I and II. Touch is mediated by mechanoreceptors in the periphery, which terminate in lamina III, IV and V. Proprioception, the perception of movement and spatial orientation, is mediated by the sensory neurons projecting through the dorsal horn to the motor neurons located in the ventral horn

1.1.3 Conduction

In the adult dorsal root ganglia (DRG), the large diameter cells contain high levels of neurofilament, producing myelinated A β fibres and a proportion of thinly myelinated A δ fibres. Indicating about 40% of lumbar DRG neurons, they express trkB and trkC (Lin et al., 2011), which are high-affinity tyrosine kinase receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively. Small diameter

cells give rise to unmyelinated axons. These cells can be differentiated histochemically into two distinct populations including those cells which constitutively synthesize neuropeptides substance P and calcitonin gene-related peptide (CGRP) and those which bind the lectin IB4 (Michael et al., 1997).

Neurophysiological studies confirm that, under normal setting, rapidly conducting A β fibres (conduction velocities $>30 \text{ m s}^{-1}$) are concerned with non-noxious input from specialized encapsulated receptors. On the contrary, most small diameter fibres, including A δ fibres (conduction velocities of $2.5\text{--}30 \text{ m s}^{-1}$) and C fibres (conduction velocities $<2.5 \text{ m s}^{-1}$), have free nerve endings and respond to noxious stimuli (Grigg et al., 1986).

The primary peripheral sensory nociceptors have their cell bodies located within the dorsal root ganglia (DRG). DRG neurons have an extended pseudounipolar structure with one end forming a sensory terminal in the skin or visceral organ, and the other end synapsing in the spinal cord. Nociceptive neurons fire action potentials in response to thermal, chemical and mechanical stimuli and possess a high activation threshold as this prevents pain from harmless stimuli. These neurons relay information from the periphery to the central nervous system (CNS).

1.1.4 Spinal transmission of pain

The pharmacology of the spinal cord is complex, such that the candidate neurotransmitters found within the CNS and their receptors are also found here. These neurotransmitters are derived from either afferent fibres, intrinsic neurons or descending fibres. The majority of neurotransmitters are located within the substantia gelatinosa, a region vital for the reception and modulation of nociceptive messages transmitted via peripheral afferents.

The peripheral afferent fibres emanating from the DRG enter the spinal cord via the dorsal roots, ending in the grey matter of the dorsal horn (Figure 3). Most of the nociceptive afferents terminate in the superficial region of the dorsal horn, the C fibres and some A δ -fibres innervating cell bodies in lamina I and II, while other A fibres penetrate deeper into the dorsal horn (lamina V). Cells in lamina I and V give rise to the projection pain pathways from the dorsal horn to the thalamus. The non-

myelinated afferent neurons contain several neuropeptides including substance P and calcitonin gene-related peptide (CGRP) which are released as mediators at both peripheral and central terminals, and play an important role in the pathology of pain. There is evidence for the involvement of the excitatory amino acids, glutamate, aspartate and peptides in nociceptive transmission within the dorsal horn of the spinal cord. Functional roles have been established where antagonists for their receptors have been developed. Some of these have been discussed below;

a) Neuropeptides

Substance P is the neurotransmitter of the small diameter nociceptive afferent terminals (Takemura et al., 2006) whose biological actions are mediated through activation of the neurokinin receptors named NK₁, NK₂ and NK₃. Substance P acting via the NK₁ receptor has been implicated in the processing of noxious information within the spinal cord, due to its involvement in animal models of inflammatory and neuropathic pain (Lagraize et al., 2010). Peripheral injection of a NK₁ agonist was shown to induce hyperalgesia in the rat hind paw (Chen et al., 2006). On the other hand, central administration of an NK₁ antagonist before and after inflammation induced by complete Freund's adjuvant, resulted in attenuation of thermal hyperalgesia (Lagraize et al., 2010).

Calcitonin gene-related peptide is a potent vasodilator (Brain et al., 1985) and is involved in pain transmission, through its ability to sensitise nociceptors (Li et al., 2008). Its role in the pathogenesis of migraine pain has been studied extensively (Arulmani et al., 2004). The antagonist BIBN4096BS was the first CGRP antagonist tested clinically. It abolished neurogenic increases in dural blood flow and was efficacious in migraine treatment (Doods, 2001). CGRP is involved in the sensitisation of pain pathways, as it is an important signalling molecule along the peptidergic nociceptive pathway from primary afferents and spinal cord to the supraspinal regions. Systematically administered CGRP antagonists BIBN4096BS dose dependently reduce inflammatory pain induced by CFA and osteoarthritic pain induced by sodium monoiodoacetate (MIA) (Hirsch et al., 2013).

b) Somatostatin

Somatostatin (SST) is polypeptide expressed in a subset of primary afferent neurons and reduces vascular and nociceptive components of inflammation. In experimental paradigms the main effect of SST receptor (SSTR) activation in the spinal cord is inhibition (Wang et al., 2009). Some studies show that central administration of SST is associated with spinal cord damage including neolysis of dorsal and ventral horn cells, pyknotic neurons, demyelination and inflammation (Gaumann and Yaksh, 1988). The rationale that targeting peripheral SSTRs would provide effective analgesia whilst avoiding the CNS side effects has been proposed. Carlton *et al.* (2001) investigated the presence of SSTRs on peripheral afferent fibres and the physiologic effects of the SST agonist octreotide on formalin-induced nociception in the rat. The results obtained demonstrated that SSTRs are present on 11% of primary sensory fibres in glabrous skin, intraplantar administration of octreotide dose-dependently reduced thermally-induced nociceptive behaviour and each of these actions can be inhibited by the SSTR antagonist cyclo-somatostatin (Carlton et al., 2001). Therefore activating peripheral SSTRs has anti-inflammatory and anti-hyperalgesic effects, while avoiding the deleterious CNS side effects and may be useful in the clinical setting.

c) Excitatory neurotransmitters

Sustained persistent activation of peripheral nociceptors due to nerve injury produced ectopic discharges in the injured sensory neurons, and subsequent release of excitatory neurotransmitters from central afferent terminals and enhance dorsal horn excitability (Dickenson, 1990). The actions of glutamate are mediated via the N-methyl-D-aspartate (NMDA) receptor and other non-NMDA receptors, which consist of metabotropic, kainate and alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Mori and Mishina, 1995). The NMDA receptor consists of two homologous subunits NR1 and NR2, which have been implicated in long-term events in the CNS such as long-term potentiation in the hippocampus, synaptic plasticity and sustained motor activity (Latremoliere and Woolf, 2009).

Activation of the NMDA receptor requires glutamate release from neurons and binding to its receptor as well as the presence of glycine (co-agonist) and the means to remove the physiological Mg^{2+} that normally block the channel, by depolarisation. This results in an influx of Ca^{2+} ions and phosphorylation of proteins. Selective NMDA antagonists are reported to reduce pain resulting from inflammation and injury to peripheral nerves (Boyce et al., 1999, Chizh et al., 2001, Kristensen et al., 1994, Murray et al., 1991).

d) Kinins

The most active kinins are bradykinin and kallidin and these are produced following tissue injury by the proteolytic cleavage of their kininogen precursor. Bradykinin is a potent pain-producing substance that acts by releasing prostaglandins, which in turn enhance the effects of bradykinin on the nerve terminal. Bradykinin and kallidin have their effects through actions on the B_1 and B_2 receptors expressed on neurons and involved in the pathogenesis of pain and inflammation. More recently it has been suggested that when inflammation is prolonged, bradykinin B_1 receptors, which are not expressed in healthy tissues to a significant degree, also play an important role in the maintenance of hyperalgesia (Dray and Perkins, 1993). Consistent with this, the non-peptide bradykinin B_2 receptor antagonist, bradyzide showed long-lasting analgesic effects in the rat model of CFA-induced mechanical hyperalgesia (Burgess et al., 2000).

e) Prostaglandins

Prostaglandins are released following tissue inflammation and damage from cells such as mast cells, macrophages and neutrophils (Syriatowicz et al., 1999) and can sensitise nociceptors (Cunha and Ferreira, 2003). They are synthesised by the enzyme cyclooxygenase 1 (COX-1) and its isoform coenzyme COX-2, which is induced in the periphery by cytokines, growth factors and other inflammatory stimuli (Ballou et al., 2000). Generally considered to be sensitising agents, they act by increasing cyclic AMP and may enhance nociceptor sensitisation by reducing the activation threshold for protein kinase A-dependent tetrodotoxin-resistant sodium channels (England et al., 1996). Studies using COX-1 and COX-2 knockout animals have

suggested that these enzymes may serve different mechanistic pathways. COX-1 deficient mice showed reduced nociception to a variety of noxious stimuli (hot plate, stretching) compared to their wild type counterparts. Measurements of mRNA revealed a compensatory up-regulation of COX-1 mRNA in the spinal cords of COX-2 null mice, but no increase of COX-2 in spinal cords of COX-1 null mice (Ballou et al., 2000).

In a model of carrageenan-induced inflammation, pre-treatment with dexamethasone (steroidal anti-inflammatory drug) prevented endogenous prostaglandins, and prevented the induction of mechanical hyperalgesia (Prado et al., 2013).

f) Cytokines

In addition to their stimulatory and inhibitory effects on immune inflammatory cells, cytokines may act directly or indirectly to sensitise nociceptors. During acute phases, cytokines appear to induce sensitisation through activation of receptor associated tyrosine kinases and phosphorylation of ion-channels, whereas in chronic inflammation, transcriptional up-regulation of receptors may play a larger role (Oprea and Kress, 2000). Administration of tumour necrosis factor alpha (TNF- α) (Wagner and Myers, 1996), interleukin-1 (IL-1), IL-6, IL-8 produce hyperalgesia, and antibodies against TNF- α reduce hyperalgesia in models of inflammatory pain (Oprea and Kress, 2000). IL-6 deficient mice show reduced response to noxious mechanical and thermal stimulation (Xu et al., 1997), following carrageenan-induced inflammatory and chronic constriction injury (CCI) neuropathic pain (Murphy et al., 1999).

g) Growth factors

Nerve growth factor (NGF) plays a key role in modulating pain sensitivity, and acts through activation of the tyrosine receptor kinase A. Administration of exogenous NGF was shown to induce thermal hyperalgesia in rodents (Andreev et al., 1995), through a mechanism involving mast cell degranulation, releasing algescic agents. Degranulation of mast cells delays the effects of NGF (Lewin et al., 1994) and attenuates CFA-induced hypersensitivity. On the other hand, sequestration of NGF

by anti-NGF antibodies prevented inflammation induced hyperalgesia (Woolf et al., 1994). In human subjects, NGF produces cutaneous hyperalgesia at the application site, and widespread pain which persist for days. It is possible that these sensitising effects are mediated by direct effects on nociceptors.

Brain derived neurotrophic factor (BDNF) is synthesised by small DRG neurons and transported within axons to the terminals in the spinal cord. BDNF in these cells is up-regulated by administration of NGF or induction of inflammation (Cho et al., 1997) and consistent with this, BDNF antiserum temporarily reduced both mechanical and thermal hyperalgesia (Theodosiou et al., 1999).

h) Other peripheral mediators

Various metabolites are released from damaged or inflamed tissue which affects nociceptive nerve terminals including extracellular ATP (Prado et al., 2013), serotonin (Abbott et al., 1996), histamine and potassium (Marker et al., 2004).

ATP excites sensory nerves by its actions on P2X₃ receptors, which are ligand gated ion channels selectively expressed by these neurons. Activation initiates a transient depolarisation by Na⁺ influx through the ion channel coupled to the P2X₃ receptor (Prado et al., 2013). Down-regulation of P2X₃ receptors by antisense oligonucleotides technologies reduces inflammatory pain (Honore et al., 2002) and P2X₃ knockout mice display significantly reduced pain-related behaviours in response to ATP or formalin (Honore et al., 2002), strengthening the evidence for a role of this P2X₃ receptor activation in acute inflammatory pain.

Injection of Serotonin (5-HT) or its agonists into the plantar surface of the hind paw causes hyperalgesia in experimental animals and sensitises nociceptors. Evidence shows that 5-HT_{1A} receptors (Millan et al., 1996), 5-HT_{2A} receptors (Abbott et al., 1996) and 5-HT₃ receptors (Voog et al., 2000, Giordano and Sacks, 1997) all contribute to the hyperalgesic action of serotonin and their antagonists could have therapeutic potential.

1.1.5 Inflammatory pain

Pain sensations could arise due to several reasons including inflammation (Linley et al., 2010). Inflammation is a complex immune response that occurs following bodily injury (wound, burn, infection, etc) which aims to remove the harmful stimulus and promote tissue regeneration. Inflammation is classified into the acute and chronic phases, each characterised by a series of cellular and biochemical reactions (Linley et al., 2010).

Acute inflammation is mediated by immune cells residing in the tissues, such as neutrophils, macrophages dendritic and mast cells. Following injury the cells release inflammatory mediators which cause inflammatory pain, by sensitising nociceptors. Inflammatory mediators involved in this process include histamine (Monroe et al., 1997), bradykinin (Burgess et al., 2000), prostaglandins (Syriatowicz et al., 1999), growth factors (Welker et al., 2000), cytokines (Cowden et al., 2010a), and substance P (Hagermark et al., 1978). These mediators result in increased vasodilatation, vascular permeability and extravasation of serum proteins and leukocytes. Histamine (Schmelz et al., 1997) and bradykinin are known to sensitise nociceptors or directly excite the peripheral terminals of nociceptive neurons

Chronic inflammation is prolonged inflammation resulting in the alteration of the types of cells present at the site of inflammation, characterised by destruction and healing simultaneously. This is caused by a variety of factors including bacterial, parasitic and viral infections, chemical irritants and non-digestible particles.

1.1.6 Neuropathic pain

Peripheral nerve injury causes both damaged and undamaged A- and C- fibres to generate spontaneous action. This is a form of ectopic output as it does not arise from the peripheral terminal and it has the ability to initiate and maintain activity-dependent central sensitisation in the dorsal horn of the spinal cord. Injured, and to a lesser extent, non-injured neurons in the dorsal root ganglion exhibit transcriptional changes that alter membrane properties, growth and transmitter

function (Xiao et al., 2002, Obata et al., 2003). Among the many changes that occur, larger fibres begin to express new neurotransmitters and modulators including substance P and BDNF, which can drive sensitisation (Fukuoka et al., 2001, Noguchi et al., 1995). Peripheral nerve injury results in degeneration of C fibre terminals in lamina II (Arvidsson et al., 1986). This loss of presynaptic input provides an opportunity for myelinated A β fibres to sprout from lamina III-IV, into lamina I-II and make contact with nociceptive-specific neurons (Lekan et al., 1996).

Peripheral nerve injury causes activation and changes in glial cells within the spinal cord as well as infiltration of peripheral immune-competent cells (Cao and DeLeo, 2008). The response is much greater than that observed during peripheral inflammation and, activated microglia synthesise and release cytokines, growth factors and neurotransmitters (Romero-Sandoval et al., 2008) and play a role in the development of pain by triggering sensitisation through actions on neurons (Raghavendra et al., 2003). Microglia release cytokines which have their effects through activation of extracellular signal-regulated kinase (ERK) (Ji et al., 2009) and cAMP Response Binding Protein (CREB) (Kawasaki et al., 2008). Activated microglia also release BDNF (Coull et al., 2003). Astrocytes are also activated following peripheral nerve injury, with a slower but prolonged time-course in comparison to microglia and may play more of a role in the maintenance of neuropathic pain and hypersensitivity than microglia (Zhang and De Koninck, 2006).

Reduced production of inhibitory neurotransmitters leads to a state of disinhibition (loss of inhibition), whose effects are similar to that produced by increases in strength of excitatory synapses and membrane excitability (Yaksh, 1989). In neuropathic pain states, disinhibition in the superficial dorsal horn with loss of GABAergic and loss of glycine inhibitory currents (Moore et al., 2002) can be attributed in part, to apoptosis of inhibitory neurons (Scholz et al., 2005). This neuronal death appears to be a result of NMDA receptor-induced excitotoxicity that develops over time, rather than the large amounts of glutamate released centrally upon injury (Scholz et al., 2005).

Another mechanism that contributes to the disinhibition observed in a subpopulation of lamina I neurons, following nerve injury is BDNF-dependent, as it modulates the anion transporter allowing changes in anion gradients across neuronal membranes to alter the inhibitory efficacy of GABA. Under physiological conditions, intracellular concentration of Cl^- are maintained by the opposing effects of Cl^- co-transporter $\text{K}^+ \text{Cl}^-$ exporter 2 channels (KCC2) and $\text{Na}^+ -\text{K}^+ -\text{Cl}^-$ exporter 1 channels (NKCC1). NKCC2 drives Cl^- ions out and NKCC1 allows influx of Na^+ , K^+ and Cl^- into the cells. The net effects of this mechanism is a steady-state Cl^- concentration gradient in which Cl^- entry into the cells hyperpolarises the neurons (Miletic and Miletic, 2008). Following nerve injury induced by ligation of the sciatic nerve, increases in BDNF released by activated microglia coincide with reduced KCC2 expression in a subset of neurons within the superficial dorsal horn (Miletic and Miletic, 2008). GABA inhibition is dependent on KCC2 activity and the loss of this activity may lead to loss of GABA inhibition within the dorsal horn. Consequently, activation of GABA receptors results in the absence of Cl^- entry into the neurons, the excitability sustained and enhanced exaggerated communication between primary afferents contributing to pain (Miletic and Miletic, 2008). The hyperalgesia caused by nerve injury was attenuated by TrkB blockers.

1.1.7 Inhibition of peripheral sensitisation

a) Opiates

Three major types of opioid receptor have been discovered and these are μ , δ and κ . Opioid receptors are G protein coupled receptors that inhibit adenylyl cyclase, thereby reducing the intracellular cAMP content. Opiates typified by Morphine, are effective in the treatment of severe acute and chronic pain but their use in the clinic has been hampered by side effects such as respiratory depression, depression, nausea, tolerance and addiction. During inflammation, expression and axonal transport of opioid receptors increases in dorsal root ganglion neurons (Stein et al., 2003). Activation of opioid receptors results in cAMP inhibition and activation

attenuates the hyperexcitability of nociceptive neurons which innervate inflamed skin. Subsequently, opioid agonists developed for peripheral use such as loperamide, show antinociceptive activity in inflammatory conditions such as experimental arthritis (DeHaven-Hudkins et al., 1999) and they have potent and long lasting analgesic effects (Decosterd et al., 2006).

b) Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs inhibit cyclooxygenase (COX) enzymes and reduce formation of prostaglandins. At present, three COX isoenzymes are known and these are COX-1, COX-2 and COX-3, which is a splice variant of COX-1 (Chandrasekharan et al., 2002). While non-selective inhibition of COX enzymes produces a significant anti-hyperalgesic effect and emphasises the importance of prostaglandins in inflammatory hyperalgesia, clinical use is limited by the gastrointestinal side effects. However, the development of selective COX-2 inhibitors has provided a means to avoid these effects. Since COX-2 is usually specific to inflamed tissue, there is less gastric irritation associated with COX-2 inhibition with a reduced risk of peptic ulceration. Etodolac, the COX-2 inhibitor was shown to attenuate heat-induced hyperalgesia in the CCI model of neuropathic pain (Suyama et al., 2004). However, analysis of clinical trial data revealed that COX-2 inhibitors are associated with a moderately increased risk of vascular events, attributable to an increased risk of myocardial infarction (Kearney et al., 2006).

c) Cannabinoids

Cannabinoids are constituents of the hemp plant and have been shown to produce a unique syndrome of behavioural effects in humans and animals that include disruption of short-term memory, cognitive impairments, and a sense of time dilation, mood alterations, and enhanced body awareness, a reduced ability to focus attention and to filter out irrelevant information, discoordination, and sleepiness. Two cannabinoid GPCRs have been discovered and they are Gi/o linked to the inhibition of adenylyl cyclase and voltage operated sodium channels causing hyperpolarisation. Initially it was believed that *CB1* receptors were predominantly located in the CNS and the plasma membrane of nerve endings and inhibits

neurotransmitter release from presynaptic terminals, whereas *CB2* receptors were non-neuronal and are mainly located in lymphoid tissue and immune cells. However, *CB1* receptors have been located in peripheral tissues such as cardiovascular and gastrointestinal tract (Szabo et al., 2001). Cannabinoids are effective analgesics in acute and chronic pain (Pertwee, 2001) and the analgesic effect is mediated by cannabinoid receptors in the dorsal horn of the spinal cord and periaqueductal grey. The antihyperalgesic efficacy of locally administered *CB1* agonist was increased because up-regulation of *CB1* receptors is induced by peripheral inflammation (Amaya et al., 2006). This indicates the possibility to develop therapeutics that target the peripheral endocannabinoid system and provide pain relief without the side effects associated with central *CB1* receptor activation (Mitrirattanakul et al., 2006).

1.1.8 Central sensitisation

Nociceptive signalling is crucial for preventing damage to the body. However, during pathophysiological conditions, this signalling may misinterpret sensory stimulation, resulting in pain. Here, the pain arises spontaneously, can be elicited by normally innocuous stimuli (allodynia), is exaggerated and prolonged in response to noxious stimuli. Sensitisation represents an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy as well as to reduced inhibition. There are two types of sensitisation, peripheral and central sensitisation. Peripheral sensitisation represents the reduced threshold and amplified responsiveness of nociceptors that occurs when the peripheral terminals of these high-threshold primary afferent neurons are exposed to inflammatory mediators and nerve injury (Chen et al., 1999) and is therefore restricted to the site of injury.

Central sensitisation on the other hand includes novel inputs to nociceptive pathway including those that do not normally drive them such as large low-threshold mechanoreceptor A β -fibre mediated pain. It also produces pain hypersensitivity in non-inflamed tissue by altering sensory responses elicited by normal inputs and increases hyperalgesia long after the initiating cause may have disappeared

(Latremoliere and Woolf, 2009). Since this is a result of alteration in the properties of neurons, the pain is no longer coupled to acute nociceptive input. The pain is generated as a consequence of changes within the CNS that alter responses to sensory inputs, rather than reflect the presence of peripheral noxious stimuli. Central sensitisation corresponds to an enhancement in the functional status of neurons and circuits within the nociceptive pathway, caused by increased membrane excitability and reduced inhibition (Latremoliere and Woolf, 2009).

Considerable numbers of substance P containing afferent fibres contain glutamate and aspartate suggesting that, both neuropeptides and excitatory amino acids are released in response to noxious stimuli. Evidence indicates that NMDA receptors influence development and maintenance of neuropathic pain (Dickenson, 1990). Gao *et al.* (2005) demonstrated an up-reregulation in the phosphorylation of the NR1 NMDA receptor (Gao *et al.*, 2005) following nerve injury. The increase in phosphorylation enhances the response of NMDA receptors to NMDA in the CNS. An increase in the amount of phosphorylated NR1 (pNR1) and pNR1 immunoreactivity in the dorsal horn neurons was observed in the ipsilateral dorsal horn spinal cord of rats with capsaicin induced secondary hyperalgesia. This supports the idea that phosphorylation of NR1 subunits is related to enhancement of synaptic efficacy and thus the development of central sensitization (Gao *et al.*, 2005).

1.1.9 Wind up

In anaesthetised rats, during repeated electrical stimulation of dorsal horn neurons with the same intensity in the periphery that activates C fibres, the initial constant response to the first few stimuli suddenly increases dramatically and then levels off at a level many times greater than the original response (Davies and Lodge, 1987). This characteristic is called wind-up because, following cessation of stimulation, the cell continues to fire for several minutes. Despite abolition or reduction of wind up in the presence of NMDA receptor antagonists, the original response of the cell remains unaltered and the cell continues to respond steadily (Davies and Lodge, 1987). Therefore, the NMDA may amplify, enhance and prolong ongoing nociceptive afferent activation and contribute to prolonged nociceptive states that underlie

central hypersensitivity (Woolf and Salter, 2000). In support of this, it has been shown that spinal responses caused by prolonged neuropathy are markedly reduced, *in vivo* by NMDA antagonists such as MK801 but not completely abolished (Smith et al., 1994).

The modulation of pain is quite complex and despite years of research, more work is required to explore the various pathways that contribute to hyperalgesia, including the histaminergic system

1.2 Histamine

Histamine [2-(4-imidazolyl)-ethylamine] has been long known to be an important mediator in different physiological and pathophysiological conditions. It is an endogenous short-acting amine synthesised from L-histidine through the catalytic activity of the rate-limiting histidine decarboxylase (HDC) (Bodmer, 1999). The classic source of histamine is the pluripotent heterogeneous basophils and mast cells, where it is stored in cytosolic granules and released via exocytosis in response to various immunological and non immunological stimuli (Kakavas S, 2006).

The diagram below shows the chemical reaction that forms histamine from the amino acid, Histidine, via catalysis with the enzyme histidine decarboxylase.

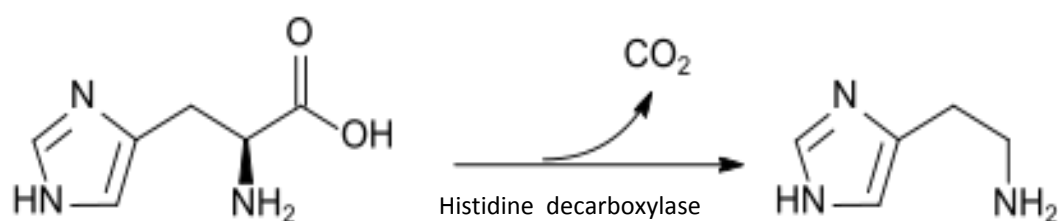


Figure 1.2 Chemical structures of the amino acid L-Histidine that forms histamine via catalysis with histidine decarboxylase (HDC) (adapted from www.histamineintoerlanz.ch)

Since its discovery over a century ago (Barger, 1910), it is one of the most studied substances in medicine possessing a spectrum of activities in immune modulation and neurotransmission. Histamine is synthesised in several types of peripheral and central tissues (Haas HL, 2008); but is found in particularly high concentrations in the lungs, skin and gastrointestinal tract.

1.2.1 Histamine in the brain

The histamine containing neurons are mainly located in the tuberomammillary nucleus (TMN) and adjacent areas within the posterior hypothalamus. The TMN is composed of several dense clusters of large, characteristic neurons, including scattered neurons with the same morphology in the surrounding heterogenous regions. Figure 1.3 represents the brain histaminergic system of all mammalian species examined. The histaminergic neurons are mainly localised in the TMN (figure 1.3), with projections throughout the brain, including the hippocampus (HPO), the basal forebrain (BF), striatum (ST) (Panula et al., 1989), hypothalamus (HY) (Meynard et al., 2005), cortex (Haas, 2003), and olfactory bulb (OB) (Panula et al., 1989).

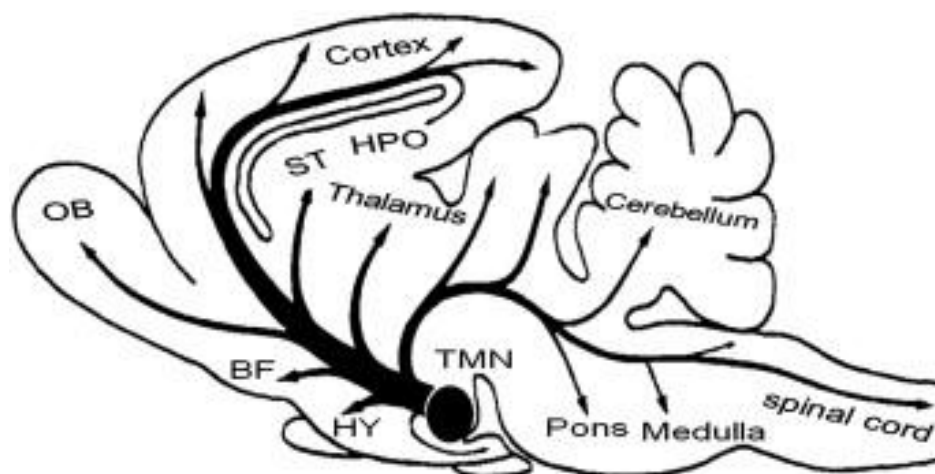


Figure 1.3: Representation of the localisation and distribution of histaminergic system in the mammalian brain. The histaminergic neurons are emanate from the TMN within the posterior hypothalamus and have projections throughout the brain (Thakkar, 2011).

The brain histaminergic system contains both neuronal and non-neuronal histamine. Histamine synthesis occurs in two steps. Firstly, it is the neuronal uptake of L-histidine by the L-amino acid transporter and secondly, decarboxylation of L-histidine by the enzyme L-histidine decarboxylase. As shown in Figure 3, once synthesised, histamine is taken up and stored in vesicles by vesicular monoamine transporter. In the absence of the high affinity uptake mechanism in the brain, released histamine is degraded through methylation by the enzyme histamine-N-methyltransferase which is located post-synaptically, and in glial cells to tele-methylhistamine. This metabolite does not possess any histamine-like activity.

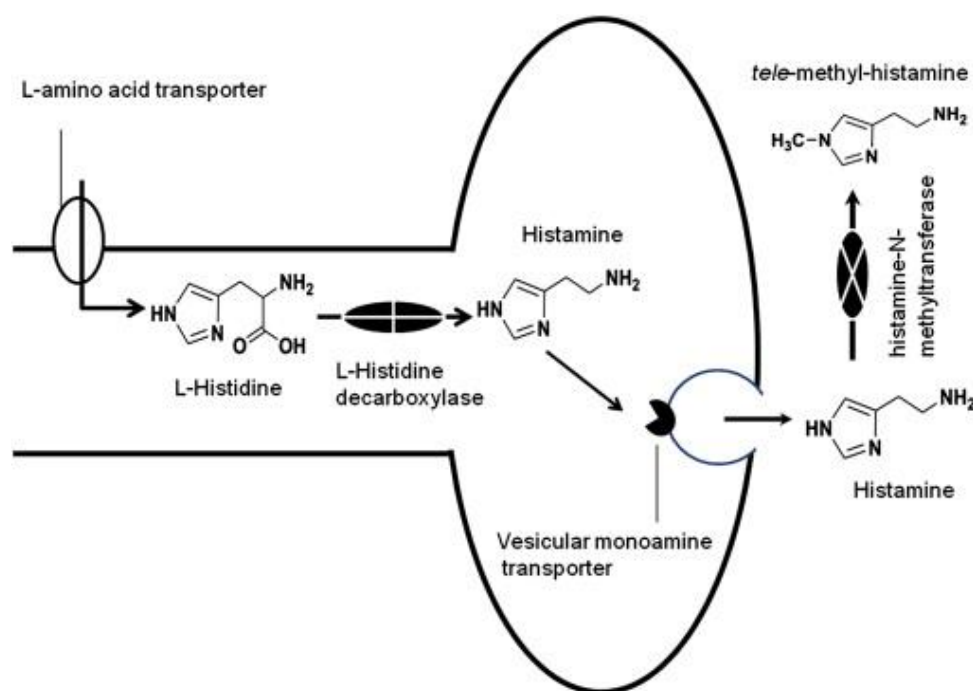


Figure 1.4 Histamine synthesis and metabolism in neurons. L-histidine is taken up by the L-amino acid transporter. The formed histamine is stored in synaptic vesicles and released by exocytosis. Free histamine in the synapse is inactivated through methylation by the enzyme histamine-N-methyltransferase (Thakkar, 2011).

The central histamine system is involved in many central nervous system (CNS) functions including, arousal (Tasaka, 1989), activation of the sympathetic nervous system, stress-related release of hormones from the pituitary (Knigge et al., 1991)

and of central aminergic neurotransmitters (Schlicker, 1994), anti-nociception (Cannon, 2003) and appetite suppression (Gotoh et al., 2007). The histaminergic system is phylogenetically conserved (Haas, 2003) and in vertebrates, the tuberomammillary nucleus (TMN), a part of the posterior hypothalamus, is the source of histaminergic neurons and their projections (Lee et al., 2008). In humans, histamine-induced itch is mediated by specific mechanoinensitive C fibres. These “itch” fibres are preferentially activated by pruritogens like histamine and react to histamine application with a time course of excitation that reflects the sensation of itch (Schmelz et al., 1997). Besides the histaminergic pathway, electrophysiological studies in humans suggest the existence of a second peripheral pathway for the transmission of itch (Schmelz, 2010).

Confirming the existence of a different itch processing pathway, itch was induced in the absence of an axon reflex flare. The axon reflex flare response comprises neurogenic vasodilation, induced by neuropeptide release from mechano-insensitive C-fibres. This suggests that itch is independent of histamine-sensitive C-fibres (Schmelz, 2010).

1.2.2 Anatomy, distribution and function of the Histamine receptors

Histamine elicits its actions through binding to four distinct histamine receptors named H₁-, H₂-, H₃- and H₄- receptors, in chronological order of discovery. All four receptors belong to the rhodopsin-like G-protein coupled receptor (GPCR) family (Simons, 2011). Homology between these receptors is very low except between H₃ and H₄ receptors. Despite this, all four contain negatively charged aspartate residue in transmembrane 3, which is considered to be the binding site for the amine function of histamine.

1.2.2.1 The histamine H₁ receptor

The first classical anti-histamines antagonised the effects of histamine on the H₁ receptor expressed on various smooth muscles and (Hill et al., 1997) is ubiquitously expressed. It has been found to exert effects on smooth muscle cells, the gastrointestinal tract, the heart, lung smooth muscle cells and the CNS, including the

cortex, cerebellum, hippocampus and thalamus. The H₁ receptor is expressed on numerous cell types including chondrocytes, hepatocytes, nerve cells, endothelial cells (Lo, 1987), neutrophils (Taniguchi, 1991) and dendritic cells (Ohtani, 2003). The presence of the H₁ receptor in the brain explains the sedative effects of the first generation anti-histamines whereas second generation anti-histamines are largely BBB-impermeable and therefore lack this effect. The H₁ receptor is linked to the G_{q/11} protein which stimulates phospholipase C (PLC) catalysed hydrolysis of membrane inositide phospholipids. Receptor activation leads to phosphatidyl-4,5-bisphosphate (IP₂) , producing inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG).

Discovery of histamine involvement in allergic reactions led to the development of anti-histamines as successful therapies for allergic and inflammatory conditions (Simons and Simons, 2011). Classic (first generation) anti-histamines have sedative side-effects and also antagonise muscarinic and serotonergic receptors (Simons, 2004). This lead to the design of second-generation anti-histamines which lack these effects and several are used therapeutically in the treatment of allergies. Examples include Loraditine (Claritin®), fexofenadine (Allegra®), cetirizine (Zyrtec®) and ketotifen (Zaditor®) have generated billions of dollars (Hill et al., 1997).

The H₁ receptor is expressed on peripheral sensory neurons and plays a role in histamine induced itch. Immunohistochemical analysis has shown the distribution of the H₁ receptor in the superficial lamina I and II of the dorsal horn of the spinal cord (Vizuite et al., 1997). H₁ receptor knockout mice show decreased inflammatory responses though loss of pronociceptive actions of histaminergic pathways in the dorsal horn (Mobarakeh et al., 2000) and intrathecal administration of H₁ antagonists elicit antinociception in a dose-dependent manner (Parada et al., 2001). *In vivo* analysis has demonstrated that intradermal administration of H₁ agonists provoke itch in mice and antagonism this receptor inhibits histamine and allergen-induced itch (Rossbach et al., 2009). Although H₁ receptor antagonists are prescribed clinically for the treatment of pruritic conditions such as atopic dermatitis, they are not always effective.

1.2.2.2 The histamine H₂ receptor

The H₂ receptor was first described in the periphery where it modulates smooth muscle and gastric acid secretion (Hill et al., 1997). The H₂ receptor is constitutively active (Leurs et al., 2002) and receptor activation is linked to the activation of adenylyl cyclase via G_s-proteins (Dy and Schneider, 2004). The H₂ receptors are expressed in the superficial lamina I and II of the dorsal horn, which receives nociceptive information.

H₂ receptors located in the periaqueductal grey are implicated in antinociception caused by administration of μ -opioids (Mobarakeh et al., 2006). A study by Farzin *et al.* (2007) showed that intraperitoneal injection of the H₂ receptor agonist dimaprit and antagonist ranitidine attenuated formalin-induced nociceptive responses in the early and late phases of the nociceptive response test (Farzin and Nosrati, 2007), suggesting that these compounds have antinociceptive effects. However, the antinociceptive effects of dimaprit were blocked by the opioid antagonist naloxone. These findings imply that central opioid receptors are involved in H₂ receptor agonist induced analgesia. This data shows that acute noxious stimuli enhance the activity of histaminergic neurons in the periaqueductal grey and dorsal horn of spinal cord which are rich in both opioid peptides and opioid receptors (Farzin and Nosrati, 2007).

1.2.2.3 The histamine H₃ receptor

The first evidence of the third histamine receptor was obtained and showed it to be a pre-synaptic auto-receptor on histaminergic neurones in the brain (Arrang et al., 1983). Subsequently, it has been shown to have pre-synaptic hetero-receptor properties in non-histamine neurones in both the peripheral and central nervous systems. Although the H₃R is mostly expressed in CNS tissues, it has been detected in the periphery (e.g. mast cells, gut) where it modulated neurotransmission (Imamura, 1995, Coruzzi, 2010). It is present on both histaminergic (auto-receptors) and non-histaminergic (hetero-receptors) and activation of the H₃ receptor can inhibit the release of histamine as well as other neurotransmitters e.g. dopamine, serotonin,

norepinephrine, glutamate, noradrenalin and γ -amino butyric acid (GABA) (Leurs, 1998).

The histamine H_3 receptor is linked to $G_{i/o}$ proteins and has been shown to be constitutive active, both *in vivo* and *in vitro* (Morisset, 2000). This receptor is pharmacologically distinct from the H_1 - and H_2 receptors, given that activation of the H_3 receptor inhibits production of cAMP via adenylyl cyclase. Autoradiographic studies with the H_3 receptor agonist [3H] R- α -methylhistamine, have shown the presence of binding sites in several rat brain regions particularly the cerebral cortex, striatum, hippocampus and olfactory nucleus, which receive ascending histaminergic projections from the posterior hypothalamus (Pollard et al., 1993). Immunohistochemical studies confirmed the existence of H_3 receptors on sensory neurons in the dorsal root ganglia and sensory fibres in the skin (Cannon et al., 2007a). The size and calibre of the neurons observed were consistent with A β and A δ fibre cutaneous innervations and were shown to be primarily medium to large DRG cells that co-expressed neurofilament and calcitonin gene-related peptide, respectively (Cannon et al., 2007a).

A subset of DRG neurons responded with an increase in intracellular calcium following stimulation with pitolisant, the H_3 receptor may mediate histamine release directly from the DRG. On the other hand, the H_3 receptor activation regulates the release of other neurotransmitters including substance p, which could activate the surrounding cells to release histamine. H_3 receptor agonists are known to inhibit neuropeptides release from presumed sensory fibres in the skin and possess some anti-nociceptive and anti-inflammatory properties (Cannon et al., 2007b, Cannon and Hough, 2005).

Systemic administration of the H_3 receptor agonist immpip significantly attenuates formalin-induced paw inflammation (Cannon et al., 2007a). Subsequently, the H_3 receptor agonist immpip attenuated formalin-induced tail flinching responses in rats (Cannon and Hough, 2005). Conversely, H_3 receptor antagonists have been shown to induce pruritus-like effects (Hossen et al., 2003). Intradermal injection of clobenpropit and histamine caused significant dose-dependent scratching behaviour

in both mast cell deficient and wild type mice (Rossbach et al., 2011). Thus injury-induced release of histamine may play a pro-nociceptive, pro-inflammatory role through action on the H₁ receptors and/or anti-inflammatory action through the H₃ receptor.

1.2.3 The histamine H₄ receptor

1.2.3.1 Gene structure and expression

The H₄ receptor is present as a single copy on chromosome 18q11.2 and homology of the H₄ receptor to the H₁ and H₂ receptors is approximately 19% (Coge et al., 2001). It was discovered after the H₃ receptor, with which it shares approximately 37% homology (Lovenberg, 1999). The gene consists of 3 exons and 2 introns. The introns divide the coding regions into three parts, encoding amino acid number 1-65, 66-119 and 120-390. This leads to the generation of alternatively spliced H₄ receptor isoforms (van Rijn et al., 2008). The open reading frame consists of 1173bp, encoding for 390 amino acid protein belonging to the family of GPCRs.

The H₄ receptor protein consists of two asparagines in the N-terminus (Asn⁵ and Asn⁹) which could function as possible glycosylation sites. Through the use of western blot analysis, Nguyen *et al.* (2001) have showed an unglycosylated (44 kDa), glycosylated (85 kDa) and putative oligomeric (>250 kDa) forms of the H₄ receptor (Nguyen et al., 2001).

Using the DNA sequence of the H₃ receptor, several research groups simultaneously and independently identified a previously unexplored GPCR sequence in the human genome as the histamine H₄ receptor (Oda, 2001, Nguyen et al., 2001, Zhu et al., 2001, Liu and Hokfelt, 2002a, Liu et al., 2001a, Morse et al., 2001). This receptor showed distinct high expression in immune cells (Morse et al., 2001, Oda et al., 2000, Zhu et al., 2001) and is now regarded as an interesting target for inflammatory disorders and pruritus (Thurmond et al., 2008b, de Esch et al., 2005).

After cloning of the human H₄ receptor, genes corresponding to the H₄ receptor were identified and cloned from mouse, rat, guinea pig, pig and monkey (Oda et al., 2002, Liu et al., 2001, Oda et al., 2005). The homology between the human and the rodent and the pig H₄ is very low (65-75%). However, the monkey and human H₄ receptor show higher homology (92%), explaining why the pharmacological profile of the human H₄ receptor is similar to that of the monkey but quite different from that of the rodent H₄ receptor. Delineating the sequences of the rat, mouse, guinea-pig, porcine, monkey and dog H₄Rs has provided a very useful tool in comparing the binding activities of the H₄ receptor ligands.

1.2.3.2 Splice variants

The H₄ receptor gene contains 2 introns, resulting in the existence of alternatively spliced isoforms. The first splice variant results in the deletion of exon 2 and encodes a protein consisting of 302 amino acids H₄R₍₃₀₂₎, from a deletion of 88 amino acids between TMII and TMIV. The second splice variant also results in the removal of exon 2. However the H₄R₍₃₉₀₎ splice acceptor site of exon 3 is recognised, leading to a frame-shift and the introduction of an alternative stop codon. The end result is a severely truncated H₄R₍₆₇₎ receptor containing only the first 67 amino acids of the H₄ receptor. Quantitative PCR showed that the H₄ receptor splice variant mRNA was most abundant in eosinophils. In pre-monocytes, expression of H₄R₆₇ is similar to that of H₄R₃₉₀. The lower abundance for mRNA of H₄R splice variants in other cell types could be due to reduced mRNA stability compared to the full length receptor.

Both the H₄R₍₃₀₂₎ and H₄R₍₆₇₎ are predominantly expressed intracellularly as determined by ELISA and western blot experiments (van Rijn et al., 2008). Although these receptor isoforms are expressed to a lesser extent at the cell surface, they are non-functional when individually expressed in recombinant mammalian cells, are not constitutively active and lack the ability to bind the agonist [³] histamine as well as the non-imidazole antagonist radioligand [³] JNJ 7777120. However, when co-expressed with the H₄R₍₃₉₀₎ the two H₄ receptor isoforms have a dominantly negative effect as they are able to decrease [³H] histamine binding to the H₄R₍₃₉₀₎ and histamine potency (van Rijn et al., 2008).

1.2.3.3 Anatomical distribution

Tissue distribution of the H₄ receptor has been profiled at mRNA level in a wide array of human and mouse tissue. In humans the H₄ receptor is expressed in abundance in bone marrow, lung, spleen, peripheral blood, heart, thymus, small intestine and colon (Liu et al., 2001a) as has been revealed by quantitative polymerase chain reaction (qPCR), northern blot, *in situ* hybridization and microarray analysis (Dijkstra et al., 2007, Dijkstra et al., 2008, Rijn, 2007, Morini et al., 2008, L. E. Sander, 2006, Sander et al., 2006). Other tissue including the stomach, liver, brain, spinal cord, kidney, placenta, trachea and the foetal brain (Liu et al., 2001a) and mouse hippocampus (Zhu et al., 2001).

Anti-H₄ receptor antibodies have been crucial to the study of the H₄ receptor and have lead to the discovery that this receptor is predominantly expressed on cells of the haematopoietic lineage such as basophils, eosinophils, T cells, mast cells and dendritic cells (van Rijn et al., 2008, Dijkstra et al., 2007, Dijkstra et al., 2008, Oda T., 2000, Oda et al., 2000) but just as important is the discovery of the receptor on non-haematopoietic cells. It has been identified in subsets of endocrine cells in the gastrointestinal tract, distinct from those containing the histamine H₃ receptor (Morini et al., 2008). Using immunological tools, the H₄ receptor has been localised in the rat gastrointestinal tract. H₄ receptor immunoreactivity was observed in the epithelium of endocrine cells in the gastric fundus (Morini et al., 2008) as well as the myenteric plexus. This provides evidence for the possible role of the H₄ receptor in the mammalian parasympathetic nervous system.

Other immunohistochemical studies have shown the presence H₄ receptor on nerves from human nasal mucosa (Nakayama et al., 2004) as well as on small and medium sized diameter neurons of the dorsal root ganglia and lamina I and II of the lumbar spinal cord (Lethbridge and Chazot, 2010b, Connelly et al., 2009, Strakhova et al., 2009). The latter results indicate the presence of the H₄ receptor on terminals of primary sensory afferent neurons. This suggests a role for the H₄ receptor in a plethora of conditions ranging from allergy and inflammation to neuropathic pain.

1.2.3.4 Histamine H₄ receptor biochemistry

H₄ receptor signalling has been studied using cells in which it is endogenously expressed as well as *in vitro* model systems such as HEK293T cells, which have been predominantly useful in deciphering the exact mechanisms as well as ligand characterisation. Similar to other GPCRs, the H₄ receptor functions as a ligand-activated guanine nucleotide exchange factor for heterotrimeric G proteins. Agonist binding stabilises the receptor's "active" conformation on the heterotrimeric G protein G α subunit thereby promoting dissociation of the GTP-bound G α subunit from the G $\beta\gamma$ subunit heterodimer (Luttrell and Gesty-Palmer, 2010). After dissociation, free G α -GTP and G $\beta\gamma$ subunits regulate the activity of enzymatic effectors such as adenylyl cyclase. Most likely G $\beta\gamma$ subunits activate phospholipase C isoforms, generating diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP₃). InsP₃ may release calcium from intercellular storage vesicles through interaction with an InsP₃ receptor.

a) Signal transduction

H₄ receptor activation results in inhibition of forskolin induced cyclic adenosine monophosphate (cAMP) production (Lovenberg, 1999). Morse *et al.* showed that the H₄ receptor exhibits a significant level of constitute activity indicated by elevated basal levels of both [³⁵S] GTP γ S binding and MAP kinase phosphorylation in H₄ receptor expressing cells, compared to wild type cells (Morse et al., 2001). Receptor activation leads to the release of the G α and G $\beta\gamma$ subunits. The G $\beta\gamma$ subunits activate phospholipase C, producing diacylglycerol (DAG) and inositol-1, 4, 5,-triphosphate (InsP₃). InsP₃ releases calcium from intracellular calcium vesicle stores, possibly through interaction with an InsP₃ receptor. The proposed mechanism of H₄ receptor activation is demonstrated in Figure 1.5.

Histamine stimulation of H₄ receptor leads to the activation of mitogen activated protein kinase (MAPK) in recombinant systems e.g. transfected HEK 293 cells, but this is absent in monocyte-derived dendritic cells (Gutzmer et al., 2005). This effect is eliminated by pre-treatment with pertusis toxin (PTX) suggesting that the receptor is coupled to G α_i G-proteins (Oda, 2001). Histamine activation of the H₄ receptor

induces calcium mobilisation from intracellular calcium stores and chemotaxis in murine mast cells; both of which can be abolished by incubation with selective H₄ receptor antagonists such as JNJ 7777120, PTX pre-treatment and inhibiting phospholipase C (PLC) using the PLC inhibitor U-73122 (Oda, 2001).

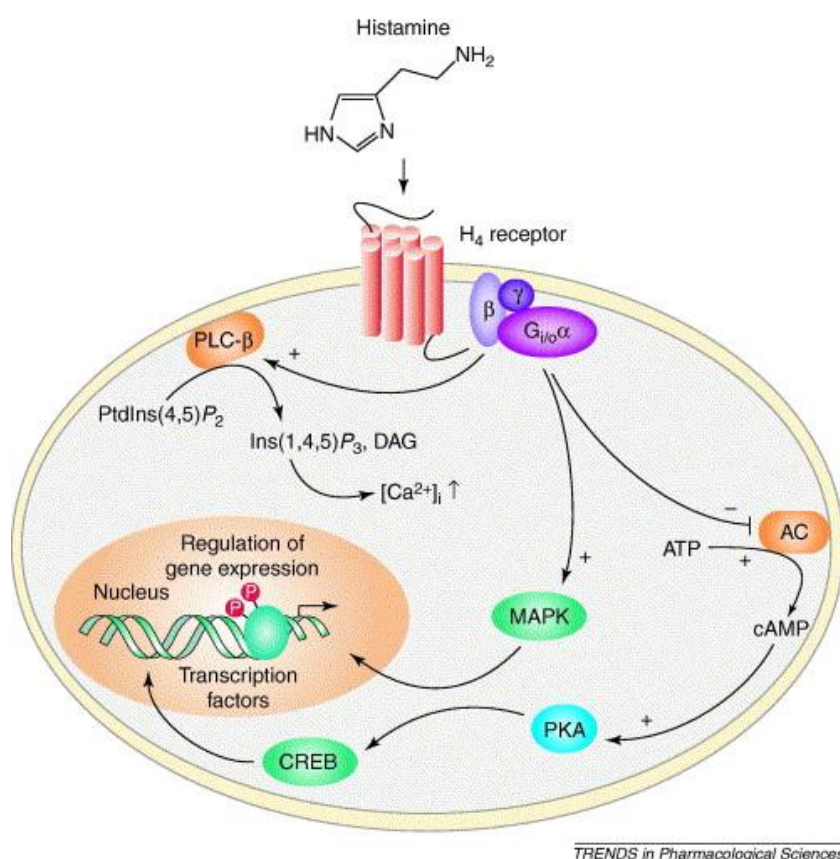


Figure 1.5 Overview of the H₄R mediated signalling (Iwan, 2005). The GPCR inhibits cAMP production via inhibition of adenylyl cyclase. The reduction in cAMP leads to a reduction in PKA activation and subsequent phosphorylation of the transcription factor CREB. The decreased PKA causes an increase in MAPK. The G_{βγ} activates PLC, which in turn hydrolyze phosphatidylinositol-4, 5-bisphosphate to DAG and InsP₃, releasing calcium from intracellular stores (Van Rijn, 2006).

Histamine induces actin polymerisation through H₄ receptor activation which is a prerequisite for cytoskeletal change in eosinophils (Barnard et al., 2008) and these effects are sensitive to PTX and are blocked by selective H₄ receptor antagonists

(Morse et al., 2001, Buckland et al., 2003). In addition, histamine and H₄ receptor agonists such as clozapine stimulate a rise in intracellular calcium, upregulation of adhesion molecules on the cell surface; CD11b and CD54 (Buckland et al., 2003) necessary for cell spreading during migration. O'Reilly *et al.* showed that histamine has a chemotactic effect on eosinophils via activation of the H₄ receptor (O'Reilly et al., 2002) whereas Buckland (2003) *et al.* were unable to do so, and these effects could be blocked by thioperamide. Nakayama *et al.* observed significant chemotaxis of eosinophils following CCL16/LEC which could be blocked using thioperamide (Nakayama et al., 2004) and pre-treatment with IL-5 resulted in a 4-fold increase in the chemotactic response of histamine (O'Reilly et al., 2002)

b) Oligomerisation

Use of biochemical and biophysical techniques has revealed that members of the GPCR family may exist as homo- and hetero-oligomers at the cell surface. Western blot analysis of tunicamycin treated cells indicates that the H₄ receptor is normally N-glycosylated. However, inhibition of N-glycosylation does not affect the presence of the putative H₄ receptor dimer. On the other hand, cross linking experiments also revealed that H₄ receptors in close enough proximity of each other are a prerequisite for oligomerisation. Despite this, N-glycosylation is not a prerequisite for dimerisation but it helps to stabilise the H₄ receptor dimers (van Rijn et al., 2006).

In vitro studies have been used to investigate the effects of ligands on H₄ receptor oligomerisation. Oligomerisation was investigated using the Bioluminescence Resonance Energy Transfer (BRET) technique, which is used to demonstrate close associations of two proteins within the cell membrane. This assay relies on the transfer of bioluminescence from a luciferase-mediated oxidation of coelenterazine, donor to a fluorescent protein acting as an acceptor. Excitation of the acceptor results in a detachable bioluminescent emission (see figure 1.6) and transfers typically occur within a radius of 100 Å (Breton et al., 2010).

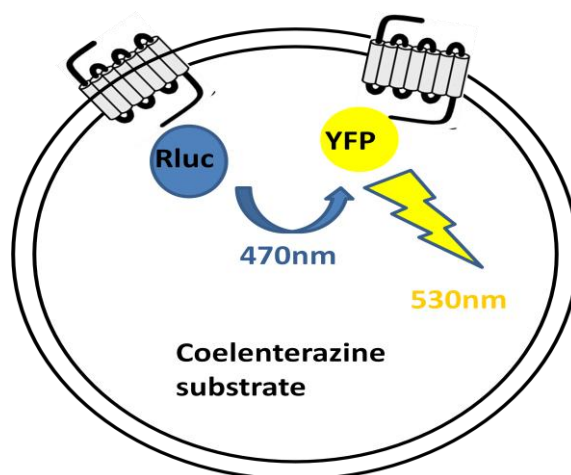


Figure 1.6 Schematic representation of the BRET assay adapted from Fiona Shenton PhD Thesis (Shenton, 2007).

In this study, BRET was performed on COS-7 cells co-expressing the H₄R-Rluc with the H₄R-eYFP (Yellow fluorescent protein). *Renilla* luciferase is fused to the C-terminal of the receptor, catalyses the emission of light from the substrate coelenterazine at a wavelength of 470 nm. This can excite the eYFP fused to another H₄R to emit light at 530 nm when in close proximity (< 100Å). A robust BRET signal confirmed the formation of H₄R homo-dimers, bringing the Rluc C-terminal within 100Å of the eYFP to allow energy transfer on addition of coelenterazine substrate (van Rijn et al., 2006). The H₄R homo-dimers are not only present in homologous expression systems, but are also present in spleen lysates endogenously expressing H₄Rs (Shenton, 2007).

Previous studies have reported that agonists can promote or reduce GPCR oligomerisation. However in the case of the H₄R, no significant difference in signal was detected when cells were treated with the H₄ receptor agonist histamine, the neutral H₄ receptor antagonist iodophenpropit or the inverse agonist thioperamide (van Rijn et al., 2006). This suggests that H₄ receptor ligands do not modulate H₄ receptor homo-oligomerisation.

1.2.3.5 Histamine H₄ receptor pharmacology

a) Agonists

Initial studies into the pharmacological and physiological functions of the H₄ receptor proved difficult due to the lack of specific ligands for this receptor, therefore early pharmacological characterisations were performed using agonists originally designed for the homologous H₃ receptor. OUP-16 was the first ligand optimised for H₄ selectivity, with a 40-fold selectivity for the H₄ receptor over the H₃ receptor (Hashimoto et al., 2003). This was followed by the description of 4-methylhistamine, which has a high affinity for the H₄ receptor while displaying a 100-fold selectivity over the other 3 histamine receptor subtypes (Lim et al., 2005). Oda *et al.* (2000) described clozapine as the first non-imidazole ligand, with anti-psychotic properties. It has been shown to possess activity at several GPCRs but almost exclusively as an antagonist (Oda et al., 2000). On the other hand, the clozapine analogue VUF6884 is also an H₄ agonist with a 10 fold higher affinity for the H₄ receptor than clozapine. Another H₄ agonist is dimaprit and its analogue VUF 8430. VUF 8430 has full agonistic activity and a 30-fold selectivity at the H₄ receptor, over the H₃ receptor (Lim et al., 2009). Together, 4-methylhistamine and VUF8430 form a useful pair of tools to study H₄ receptor pharmacology (Lim et al., 2009).

b) Antagonists

The first H₄ receptor antagonists used to characterise the H₄ receptor were imidazole-containing dual H₃- and H₄ receptor compounds (Gbahou et al., 2006) with the most important being the H₃- antagonist and H₄ receptor inverse antagonist, thioperamide (Buckland et al., 2003, Hofstra et al., 2003). The identification of JNJ 7777120 as a potent and highly selective antagonist at the human H₄ receptor marked the initiation of investigations into the roles of the H₄ receptor. JNJ 7777120 binds to the H₄ receptor with high affinity (K_i, 4 nM) and has at least 1000-fold selectivity over the H₁- or H₃ receptors (Fung-Leung et al., 2004). JNJ 7777120 has been utilised extensively to elucidate the roles of the H₄ receptor in various allergic

and inflammatory processes (Coruzzi et al., 2012), including chemotaxis of eosinophils (Ling et al., 2004) and mast cells (inhibition of chemotaxis and calcium mobilisation) (Fung-Leung et al., 2004), plus allergic rhinitis (Takahashi et al., 2009) and allergic airway inflammation (Dunford et al., 2006). Although it has a reasonable bioavailability (22%), JNJ 7777120 has a very short *in vivo* half life in rats (0.8 hours) (Jablonowski et al., 2003). Overall, these data demonstrate that JNJ 7777120 has anti-inflammatory properties which suggest that H₄ receptor antagonists may possibly constitute a new class of anti-inflammatory drugs. However, care needs to be taken when interpreting data because JNJ 7777120 is an antagonist at the human H₄ receptor, an agonist at the macaque, dog, guinea-pig and rat H₄ receptor and an inverse agonist at the mouse H₄ receptor (Liu et al., 2001). Therefore the effects of drug administration could be specific to the pathway that is activated. A direct analogue of JNJ 7777120 known as JNJ10191584 (VUF 6002) is another potent and highly selective H₄ receptor antagonist.

PF-3893787 was shown to inhibit imetit induced eosinophil cell shape change (Mowbray et al., 2011). Mowbray *et al.* (2011) showed that PF-3893787 is an inverse agonist at the human and macaque H₄ receptor, antagonist at the dog H₄ receptor and partial agonist at guinea pig and rodent H₄ receptors (Mowbray et al., 2011).

c) Biased signalling

JNJ 7777120 has been shown to act as a biased agonist at the H₄ receptor, acting as a neutral agonist with [³S] GTPγS binding and a partial agonist with β-arrestin recruitment (Rosethorne and Charlton, 2011). β-arrestin belongs to a small family of proteins that are essential for the regulation of signal transduction. G protein coupled receptor kinase (GRK) phosphorylation prepares the activated (agonist occupied) receptor for β-arrestin binding and this prevents further G protein mediated signalling, targets the receptor for internalisation and redirects signalling to an alternative pathway independent of G proteins. Studies of GPCRs like the β-adrenergic receptor have revealed that receptor activation promotes the translocation of β-arrestin from the cytoplasm to the cell membrane and subsequent

interaction of the β -arrestin with the activated receptor (Shenoy and Lefkowitz, 2005b). Though traditionally, β -arrestin recruitment to the 7-TM has been associated with receptor-uncoupling from the G proteins and desensitization, it has been recently found that β -arrestin can also serve as a signal-transducing protein with the ability to stimulate G protein independent pathways such as ERK (Luttrell and Gesty-Palmer, 2010, Shenoy and Lefkowitz, 2005a).

This phenomenon has been attributed to the existence of multiple active receptor conformations, with the agonist being able to stabilise a subtly altered state that may preferentially couple to one pathway over another. The data of Rosethorne and Carlton (2011) show that in the osteocarcinoma cell line (U2OS), JNJ 7777120 is able to stabilise a conformation in human H_4 receptor that induces β -arrestin recruitment and stimulates a down-stream signalling pathway. Therefore, JNJ 7777120 can no longer be considered the “gold standard” H_4 receptor antagonist, but depending on the parameter used, it may also act as an agonist (Rosethorne and Charlton, 2011).

1.2.3.6 Potential clinical applications and histamine H_4 receptor targeted therapies

H_4 receptor agonists and antagonist have an important role to play in the clarification of the (patho) physiological role of the histamine H_4 receptor, as well as explore its therapeutic in human disease.

a) Asthma

Asthma is characterised by airway hyper-responsiveness and chronic inflammation of the airways. A role for the H_4 R in a murine ovalbumin-induced inflammation model of asthma was discovered by Dunford *et al.* This study showed that H_4 R antagonism by JNJ777120 reduced airway hypersensitivity and Th2 cytokine levels in diseased lung tissue, marked by attenuation of IL-13 driven pathologies (Dunford et al., 2006) such as airway remodelling. In a different study, UR-60427 a selective and high affinity H_4 R inverse agonist showed high efficacy and potency in a rat asthma model (Alfon et al., 2010). In contrast, the selective H_4 R agonist, 4-Methylhistamine

reduced airway hyperactivity, airway resistance and inflammation in a mouse model of allergic inflammation. This effect was attributed to increased migration of CD4⁺ CD25⁺ FoxP3⁺ Tregs (T regulatory) to the site of inflammation, where accumulated T cells released large amounts of the anti-inflammatory cytokine IL-10 (Morgan et al., 2007). Furthermore, it has been reported that JNJ 7777120 decreased the levels of IL-17 in a mice asthma model (Dunford et al., 2006). However, these published data were for human Tregs and not their murine counterparts so it cannot be ruled out that human and mouse Tregs respond differently to stimulation of H₄R.

b) Autoimmune disorders

Histamine has been implicated in the pathology of autoimmune diseases including Rheumatoid arthritis (RA), characterised by synovial tissue inflammation and subsequent cartilage erosion leading to joint deformity. Localisation of the H₄ receptor in vascularisation and cell walls of patients with RA (Grzybowska-Kowalczyk et al., 2007) and osteoarthritis (Grzybowska-Kowalczyk et al., 2008) and the identification of H₄ receptor in fibroblasts and macrophage-like cells from RA synovial tissues (Ohki et al., 2007) support the contribution of the receptor in the pathophysiology of the disease. The antinociceptive effects of JNJ 7777120 in a rat model of osteoarthritis provides evidence that selective H₄ receptor antagonists may be an attractive approach for the development of new drugs for the treatment of osteoarthritis pain (Hsieh et al., 2010a).

Multiple sclerosis is an autoimmune disease of the CNS characterised by demyelination and axon loss and gliosis. Though the specific aetiology remains undefined, susceptibility is thought to result from genetic/epigenetic, environmental, immunological, hormonal and infectious agents. Experimental allergic encephalomyelitis (EAE) is the animal model of MS in which disease pathogenesis is associated with MHC Class II CD4⁺ T cells with the ability to secrete IFN γ and IL-17. Histamine modulates blood brain barrier permeability and enhances leukocyte rolling as well as vascular extravasation of leukocytes into the CNS. Studies have recently shown a role for H₄R in modulation of the immune system during EAE. Saligrama *et al.* (2012) showed that H₄RKO C57/BL6 and H₃H₄ receptor knockout

mice presented a more severe EAE pathogenesis, augmented neuroinflammation and increased blood brain barrier permeability compared to the WT and H₁H₂ receptor knockout littermates respectively (Saligrama et al., 2012). The study also showed that H₄ receptor signalling controls the frequency of regulatory T cells (T_{reg}) in the CNS, including chemotaxis and the suppressive ability of the T regulatory cells (del Rio et al., 2012). These findings suggest that the use of peripheral and central acting H₄ receptor agonists may be useful in treating patients with MS at the onset or upon relapse.

Histamine has also been implicated in the Sjogren's syndrome (SS), also known referred to as 'autoimmune epithelitis'. This is because the tubuloacinar epithelial cells are believed to form auto-antigens. Symptoms of the disease include dry mouth and dry eyes due to immune cells attacking the salivary gland and tear ducts. The H₄ receptor was shown to be functionally expressed in human salivary glands and expression was reduced in patients with SS (Stegaev et al., 2012). This was consistent with histamine producing cells stimulating the H₄ receptor-positive tubuloacinar cells, leading to receptor down-regulation via clathrin-mediated endocytosis (Stegaev et al., 2013). This suggests that H₄ receptor may play some constitutive role in the maintenance of healthy salivary epithelium, a function which, based on changes in local histamine synthesis and/or greatly diminished H₄R levels, is disturbed in SS (Stegaev et al., 2012).

c) Cancer

H₄ receptor expression was shown to be down-regulated in colorectal cancers and this correlated with tumour progression (Boer et al., 2008). Down-regulation of H₄ receptor in gastric carcinomas plays a role in histamine-mediated growth control of GC cells. Melanoma cells express the H₄ receptor at the messenger RNA and protein levels. Using histamine agonists, antagonists, and H₄ receptor small-interfering RNA the inhibitory effect of histamine on proliferation was in part mediated through the stimulation of the H₄ receptor and the decrease in proliferation was associated with an induction of cell senescence and an increase in melanogenesis, a differentiation marker of these cells (Massari et al., 2011). Another study showed that the H₄

receptor agonist clobenpropit suppressed human cholangiocarcinoma progression by disrupting epithelial mesenchymal transition and tumour metastasis (Meng et al., 2011). Interruption of invasion and tumourigenesis by histamine may add to therapeutic advances for cholangiocarcinoma.

d) Gastrointestinal disorders and inflammatory bowel disease

H₄ receptor expression was detected by immunohistochemistry in different areas of the gut (Chazot 2007, Boer, Helinger et al. 2008) and various studies have revealed the protective effect of the antagonist JNJ 777120 in several models of gastric and intestinal damage (Coruzzi 2010). JNJ 777120 has protective effects against indomethacin-induced gastric lesions in rats and also prevented the aggravating effects of the H₃ receptor antagonist immapip (Coruzzi et al., 2011). This information suggests a potential ulcerogenic role for the H₄ receptor and there could be therapeutic value for H₄ receptor targeting drugs in gastric pathology.

Selective H₄ receptor antagonists have been evaluated in the widely used experimental model of inflammatory bowel disease provoked by colonic instillation of the hapten, trinitrobenzene sulphonic acid. JNJ10191584 and JNJ 777120 significantly attenuated hapten induced macroscopic injury, score and fall in body weight (Varga et al., 2005). Both compounds dose-dependently reduced elevated levels of colonic myeloperoxidase and neutrophil influx. TNF α levels were reduced by treatment with JNJ10191584 and JNJ 777120 (Varga et al., 2005) and it is possible that TNF- α level reduction reflects an action of the H₄ receptor antagonists on the cellular transduction mechanisms promoting cytokine biosynthesis. This suggests a possible pro-ulcerogenic role of histamine H₄ receptor in the rat gastric mucosa. The findings of a protective effect of H₄ receptor antagonists could point to a novel pharmacological approach to the therapy for gut inflammation.

e) Allergic rhinitis

H₄ receptor expression was significantly increased in the nasal mucosa of allergic rhinitis patients in both structural and immune cells, compared to healthy controls (Yan et al., 2010a). In a separate study, a H₄ receptor antagonist was shown to

relieve the symptoms and inflammatory conditions of allergic rhinitis (Yan et al., 2010b) though the effect was weak in comparison to that of Loratadine. In contrast JNJ 7777120 significantly inhibited nasal symptoms and exhibited immunomodulatory functions in a mice model of allergic rhinitis (Takahashi et al., 2009).

Palau Parma has developed the first reported H₄ receptor antagonist for the treatment of allergic respiratory disorders. The compound, UR-63325 has finished the phase I first-in-man clinical trial with promising interim data (Sisniega, 2010).

f) Central Nervous System effects

Evidence has shown that histamine release is a sensitive indicator of stress and the involvement of the histaminergic system in the modulation of anxiety behaviours in animals has been suggested. All four histamine receptors (H₁-H₄) are expressed in the brain. H₄ receptor expression in the brain has only recently been reported but its function has not been elucidated. In a behavioural test using H₄ receptor KO BALB/C mice, the KO mice presented an enhanced escape response to approach and struggled more when touched or restrained by the tail compared to the WT. On the other hand, the KO mice showed better performance in the Morris water maze test compared to the WT. This study revealed that there are differences in the behavioural phenotypes of the H₄ receptor KO mice, but further studies are required to analyse the differences (Rossbach et al., 2012).

The administration of histamine induces an anxiogenic effect and destruction of the rat TMN region, from which histaminergic neuronal fibres arise, can induce an apparent anxiolytic effect (Frisch et al., 1998). The effect of the H₄ receptor agonist VUF 8430 on anxiety in the light dark box test was recently reported. Galeotti *et al.* (2013) demonstrated that intracerebroventricular (i.c.v) administration of this VUF 8430 produced a dose-dependent increase in the time the mice spent in the light compartment. The “light-dark” test exploits the conflict between the animal’s tendency to explore a new environment and its fear of bright light. These results indicated the induction of an anxiolytic effect comparable to that exerted by diazepam. The effect of VUF 8430 was abolished by pre-treatment with the H₄ receptor antagonist JNJ 10191584 (Galeotti et al., 2013).

Histamine has been implicated in learning and memory. Antagonism of pre-synaptic H₃ receptors would enhance release of histamine and other neurotransmitter systems involved in cognition and indirectly ameliorate cognitive performance (Esbenshade et al., 2008). The effect of neuronal H₄ receptors on the modulation of memory processes was investigated by Galeotti *et al.* (2013) in a model of scopolamine induced amnesia. Pre-treatment with the H₄ receptor agonist VUF 8430 was shown to prevent scopolamine induced memory impairment by enhancing the time taken to enter the dark compartment of the box. These results indicate a positive effect on neuronal H₄ receptor activation on memory impairment, further clarifying the role of the histaminergic system on cognitive functions.

A role for brain histamine on feeding behaviour has been postulated. The modulation of neuronal H₄ receptors was observed in mice deprived of food for 12 hours; these conditions were needed to highlight reduction in food consumption. Intracerebroventricular administration of the H₄ receptor agonist VUF 8430 significantly reduced food consumption, demonstrating an anorexant effect (Galeotti et al., 2013). However, more tests using other agonists and antagonists are required to clearly define the role of the H₄ receptor in the regulation of appetite and satiety.

g) Pruritus

Pruritus is experienced as an unpleasant sensory and emotional sensation associated with an actual or perceived disruption to the skin that produces a desire to scratch (Magerl, 1996). Acute itch is a daily occurrence normally abolished by brief scratching near the affected area. However, chronic itch is debilitating and brief scratching provides no relief. Pruritus associated with chronic diseases such as atopic dermatitis is poorly controlled clinically due to its' significant negative impact on the quality of life of the patient.

Itch is reliably induced by a variety of chemical stimuli including kinins (e.g. bradykinin), neuropeptides (e.g. substance P), enzymes but the prime of which is histamine acting on the histamine H₁ receptor located on nociceptive neurons. However, their effects appear to be largely mediated by histamine because the

magnitude of itch is significantly diminished by administration of H₁ receptor antagonists. Histamine is the main mediator of itch and it acts through H₁ receptor activation. This was discovered as the presence of histamine sensitive central projection neurons responding to histamine iontophoresis and differed from other pain processing neurons in their central thalamic projections and resting activity (Craig, 2003). Although H₁ receptor antagonists are effective in reducing experimental histamine-induced itch, they are ineffective in the clinical treatment of pruritus associated with chronic diseases like atopic dermatitis (Hong et al., 2011). In recent years, the roles of the H₁ receptor and H₄ receptor in pruritus have been documented. In mice, the selective H₄ receptor agonist 4-Methylhistamine has been shown to induce itch in a dose dependent manner, similar to histamine (Dunford et al., 2007). This was significantly inhibited by dose-dependent administration of JNJ 7777120, and this compound had a far superior effect on the attenuation of experimental pruritus and only the centrally acting H₁ receptor antagonist diphenhydramine showed anti-pruritic activity (Dunford et al., 2007). On the other hand, this pruritic response could not be completely inhibited by the administration of either H₁ receptor antagonist or H₄ receptor antagonist alone, however when both antagonists were administered simultaneously, they had a synergistic effect in the complete abolition of scratching behaviour.

Histamine is the main pruritogen released by mast cell degranulation. Mast cells release other pruritogens including substance P, which is a neuropeptide with the capacity to sensitise free nerve endings. In a study by Yamaura *et al.* (2009), histamine H₄ receptor inhibition by orally administered JNJ 7777120 was shown to have a greater inhibitory effect on histamine induced pruritus, compared to fexofenadine, the selective histamine H₁ receptor antagonist (Yamaura et al., 2009). Similarly, pruritic responses to intradermally induced substance P-induced were observed. In this study, the non-centrally acting H₁ receptor antagonist fexofenadine had no effect on substance P-induced scratching (Yamaura et al., 2009). In contrast, oral dosing of JNJ 7777120 reduced substance P-induced scratching in a dose-dependent manner (Yamaura et al., 2009).

A study by Suwa *et al.* (2001) utilising a model of trinitrochlorobenzene (TNCB) induced chronic atopic dermatitis in sensitised hairless mice, JNJ 7777120 had a dose-dependent inhibitory effect on TNCB-induced pruritus and mast cell accumulation (Suwa *et al.*, 2011). On the other hand, the H₁ receptor antagonist fexofenadine has no suppressive effects on the scratching bouts. In this regard, pruritus induced by TCNB is most likely regulated by other histamine receptors, namely the H₄ receptor.

The evidence derived from these studies suggests that H₁ receptors have limited involvement in histamine-induced pruritus. The significant effects of the H₄ receptor antagonist JNJ 7777120 suggest that histamine may have an involvement in diseases such as atopic dermatitis via the H₄ receptor. Though this compound crosses the blood brain barrier, it lacks sedative effect and therefore, its pruritic inhibitory actions are not secondary to sedation (Dunford *et al.*, 2007).

Interestingly, itch-inducing properties of 4-methylhistamine and inhibitory effects of JNJ 7777120 were unaltered in mast cell deficient mice (Dunford *et al.*, 2007). Due to these findings, a possible presence of the histamine H₄ receptor on sensory neurones and the use of antagonists in the amelioration of skin inflammation and pruritus in patients with dermatitis were postulated.

h) Pain

The selective H₄ receptor antagonist JNJ 7777120 was shown to readily cross the BBB and exert robust anti-hyperalgesic effects on several animal models of inflammatory and neuropathic pain (Hsieh *et al.*, 2010a). The *in vivo* inflammatory pain models used include the carrageenan-induced acute inflammation, complete Freund's adjuvant-induced chronic inflammation, zymosan-induced peritonitis and the knee-joint osteoarthritis model. This data suggests that the H₄R has a role in inflammatory pain in rats and it is possible that the anti-nociceptive effects of the H₄R antagonists could be secondary to the anti-inflammatory actions of the antagonists. Hsieh *et al.* (2010) provided the first evidence of the effectiveness of JNJ 7777120 as an analgesic in the reduction of chronic constriction injury of the sciatic nerve and spinal nerve ligation models of induced neuropathic pain.

1.2.4 Conclusions and Aims

Histamine is available in many parts of the body and histamine receptor distribution is widespread. In the periphery, histamine mediates inflammatory and immune reactions, as well as control of vascular permeability, smooth muscle contraction and gastric secretion. Histamine in the CNS plays a role in the maintenance of homeostasis through regulation of arousal, the sleep-wake cycle, feeding behaviour, learning and memory. This is crucial for brain development, physiology and pathophysiology, danger recognition, and survival. Histamine interacts both directly and indirectly with a variety of receptors and chemical messengers to allow the body to respond to different situations allowing an integrated response to multiple influences.

Molecular biology has rapidly increased our understanding of the cell signalling, progression of H₄R ligands into the clinic has been limited by the variety of actions elicited by the H₄R, depending on the anatomical, physiological and biochemical properties in cells as well as pathological and physiological conditions. Due to differential G-protein coupling, H₄ receptor ligands may behave variably in different expression systems and animals species. Despite this, cloning of the H₄ receptor has led to a rapid increase in our understanding of its structure and function, development of better experimental ligands with consistent *in vivo* and *in vitro* characteristics is required. Pre-clinical data supports the potential of the H₄ receptor as a new drug target in inflammatory conditions. Our laboratory developed a unique anti-human (Shenton, 2007) and anti-mouse H₄R (Lethbridge and Chazot, 2010b) antibody and this has been used to map H₄R distribution in CNS and immune cells.

The major hypotheses addressed in this PhD thesis:

1. H₄Rs are expressed on primary sensory afferents.
2. H₄Rs are differentially expressed in the periphery and central nervous system in different pain states.

3. Antagonism of the H₄R with the antagonist JNJ 7777120 ameliorates central inflammation observed during experimental autoimmune encephalomyelitis (EAE)
4. Antagonism of the central H₄R with JNJ 777120 has anxiolytic effects and improves memory performance in mice

To test these hypotheses firstly, the previously validated anti-hH₄R antibody (Fiona Shenton, PhD Thesis (Shenton, 2007)) was be used to confirm the expression of H₄Rs on primary sensory afferents in the periphery as well as the dorsal horn of the spinal cord where they terminate. The next step was be to investigate functional expression of the neuronal H₄R using the selective H₄R agonist VUF 8430, in a dorsal root ganglia (DRG) model of calcium signalling. An additional aim was to probe the potential differential expression in acute inflammatory and neuropathic pain conditions. The final step will probe the effects of H₄R antagonism using selective ligand, JNJ 7777120 on the mouse model of experimental allergic encephalomyelitis (EAE) and on the behaviours of mice in a series of novel *in vivo* tests.

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1. Source of materials

2.1.1 Sigma-Aldrich chemical company (Poole, Dorset, UK)

Folin-ciocalteu phenol reagent.

Pre-stained molecular weight marker (molecular weight range 26-180kDa).

Tween-20.

p-coumaric acid.

Luminol.

Hydrogen peroxide (30%v/v).

Kodak D-19 developer.

Kodak fixer.

Ampicillin

Agarose.

Agar.

Dulbecco's modified eagle medium/F12

Dulbecco's modified eagle medium, D5671

L-glutamine (200mM)

7.5% sodium bicarbonate

Sodium hydroxide.

Trypsin 0.5g, EDTA 0.2g per litre of Hanks.

Ethylenediaminetetracetic acid (EDTA).

Ethylenebis(oxyethylenenitrilo)tetracetic acid (EGTA).

Sodium phosphate.

Sodium azide.

Sodium citrate.

Triton X-100.

Anti- β -actin.

Dithiothreitol (DTT).

Dialysis tubing (visking size 11/4").

Sodium dodecyl sulphate (SDS).

B-mercaptoethanol.

Bromophenol blue.

Glycine.

Phosphate buffered saline.

Foetal bovine serum

Poly-D-Lysine

HAT media supplement (50x Hybri-Max) [™]

Phosphate buffered saline solution. 10x concentrate.

Dibutyl cyclic AMP (db-cAMP)

Magnesium chloride

Hepes

Histamine

2.1.2 Fisher scientific

Acrylamide/bis-acrylamide 30%.

Tris

Methanol.

Sodium chloride (NaCl).

N, N, N', N' –Tetramethylethylenediamine (TEMED)

Calcium chloride

2.1.3 Amersham International (Aylesbury, Bucks, UK)

Blotting paper.

Nitrocellulose.

Hyperflim™.

HRP linked secondary antibody-rabbit.

HRP linked secondary antibody-mouse.

Binding filters

2.1.4 Tocris (Bristol, UK)

JNJ 7777120

Sigma-Genosys (Cambridge, UK)

Histamine H₄R peptide.

Chazot 3 human H₄ (374-390) C-terminal CIKKQPLPSQHRSVSSS

2.1.5 QIAGEN Ltd (Dorking, Surrey, UK)

QIAGEN® plasmid maxi kit.

2.1.6 Abcam (Cambridge, UK)

Mouse anti-Calcitonin gene-related peptide (CGRP)

Guinea pig anti-Substance P

Mouse anti-CD11c

2.1.7 Invitrogen (Paisley, UK)

Alexa Fluor 594 Donkey anti-rabbit

Alexa Fluor 488 Goat anti-mouse

Fluo-4 AM

2.1.8 BDH Laboratory Supplies (Poole, UK)

Ammonium peroxodisulphate (APS)

1,4-Dithiothreitol (DTT)

N,N,N',N'-teramethylethylenediamine (TEMED)

Methanol

Glycerol

Isopropanol

Ethanol

Dimethyl Sulphoxide (DMSO)

Sodium hydrogen carbonate

Potassium phosphate

Hydrochloric acid

Citric acid

Sodium chloride

Acetic acid

DPX mountant

Potassium chloride

Diethylamine

Glucose

2.1.9 Jackson ImmunoResearch (Suffolk, UK)

Texas-red Donkey anti-rabbit.

FITC-conjugated secondary goat anti-mouse.

2.1.10 Cambrex Bio Science (Verviers, Belgium)

Foetal calf serum

2.1.11 Miscellaneous

Human H₄ cDNA was a gift from Professor Rob Leurs (Free University, Amsterdam, The Netherlands).

VUF 8430 was a gift from Professor Holger Stark (University of Dusseldorf, Germany)

Human embryonic kidney (HEK) 293 cells were obtained from the European collection of cell cultures, Salisbury, Wilts.

2.2 Instruments and Equipment

Spectrophotometry: Jenway Genova Spectrophotometer

Centrifuges: An eppendorf centrifuge, 5415R was used for all volumes less than 1.5 ml. A refrigerated Harrier 18/80 EPS 301 was used for falcon tubes of volumes 15 ml or 50 ml.

Incubators: shaking incubator, cell incubator Shel Lab (Sheldon Manufacturing Inc.).

Mini Orbital shaker: Stuart scientific 505.

Water bath: Nuve bath.

Heat block: QBT2 heating block (Grant).

Magnetic Stirrer/Hot plate: Stuart Scientific, Velp Scientific

Vortex: Scientific Industries vortex-2 genie

Rocker: Grant-Bio PMR-30

Roller Mixer: A Stuart scientific roller mixer SRT2 was used for all falcon tubes with volumes of 15 ml or 50 ml.

Balances: Milligram amounts were weighed using a Mettler Toledo classic. All other amounts were weighed using a Scouts Pro balance (Ohaus).

Electrophoresis equipment: Polyacrylamide gels were cast in Hoefer SE 245 dual gel caster using gel plates of 10x8cm. Electrophoresis was performed using a Hoefer mini-vertical gel electrophoresis unit SE260 and transferred using a Hoefer TE 22 tank transfer unit, power was from an Electrophoresis Power Supply EPS 301, all supplied by Amersham Biosciences.

Microscopes: Zeiss Axioskop 2

Nikon eclipse TE-2000E

Photography: Nikon digital camera used for immuohistochemistry

Other equipment: immunoblotting cassette, pH meter was a Mettler Toledo MP220, automatic pipette man.

Glassware, plastics and disposables: Hamilton syringe. Dounce glass/glass homogeniser. Cell scrapers, 250 ml sterile cell culture flasks, petri dishes and sterile pipettes from Greiner. Sterile filters: 0.2 μ m Sartorius Sartolab-V150 filter unit. Cryogenic vials. Sterile pipettes and 250ml sterile filter lid cell culture flasks from Greiner. Falcon tubes. Columns. Eppendorf tubes, and pipette tips from Starlabs, UK. Glass slides and cover-slips from VWR.

2.3. Preparation of standard solutions

2.3.1 Lowry reagent A:

2% (w/v) sodium carbonate, 0.1 M sodium hydroxide and 5% (w/v) SDS

2.3.2 Lowry reagent B:

2% (w/v) sodium potassium tartrate.

2.3.3 Lowry reagent C:

1% (w/v) copper sulphate

2.3.4 Stacking gel buffer:

0.5 M Tris-glycine, pH 6.8 containing 8 mM EDTA and 0.4% (w/v) SDS.

2.3.5 Resolving gel buffer:

50 mM Tris, 384 mM glycine, 1.8 mM EDTA, pH 8.8 and 0.1% (w/v) SDS.

2.3.6 Stock acrylamide:

40% (v/v) acrylamide and N,N'-methylenebisacrylamide.

2.3.7 Electrode buffer:

50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS pH 8.8

2.3.8 Sample buffer:

30 mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS.

2.3.9 Pre-stained molecular weight markers:

Pre-stained molecular weight marker stored in sample buffer

2.3.10 Ammonium Peroxodisulphate (APS):

1 mM in dH₂O

2.3.11 Dithiothreitol (DTT):

200 mM in dH₂O

2.3.12 Transfer buffer:

25 mM Tris, pH 8.4, 192 mM glycine and 20% (v/v) methanol.

2.3.13 Tris buffered saline:

50 mM Tris-HCl, 0.9% NaCl pH 7.4.

2.3.14 Tris/EDTA (TE) buffer:

10 mM Tris, 1 mM EDTA, pH 8.0.

2.3.15 Homogenisation buffer:

50 mM Tris-HCl pH 7.4, containing 5 mM EDTA and 5 mM EGTA.

2.3.16 H₄ receptor binding buffer:

50 mM Tris-HCl, pH 7.4

2.3.17 Immunoblotting blocking buffer:

5% marvel (w/v), 0.2% tween 20 (v/v) and TBS pH 7.4

2.3.18 Immunoblotting incubation buffer:

2.5% marvel (w/v), TBS pH 7.4

2.3.19 Immunoblotting wash buffer:

2.5% (w/v), 0.2% (v/v) tween 20, TBS pH 7.4

2.3.20 ECL Developing solution A:

1 ml 250mM luminal, 0.44 ml 90 mM coumaric acid, 10 ml 1 M Tris-base pH8.5.

Make up to 100 ml with dH₂O

2.3.21 ECL Developing solution B:

64 µl 30% H₂O₂, 10ml 1 M Tris-base, pH 8.5. Make up to 100 ml with dH₂O

2.3.22 Kodak developing solution:

100 ml stock developer and 400 ml dH₂O. Photosensitive therefore cover tub.

2.3.23 Kodak fixative solution:

125 ml stock fixer and 37 5ml dH₂O. Photosensitive therefore cover tub

2.3.24 HEK293 freezing media:

70% foetal bovine serum, 20% DMEM, 10% DMSO

2.3.25 Preparation of HEK293T cell growth media:

DMEM, 10% (v/v) foetal bovine serum, 2 mM L-glutamine. Media was filtered using a 0.2 µm Sartorius Sartolab V150 filter unit and stored at 4⁰C until use.

2.3.26 Preparation of F11 cell growth media:

DMEM, 15% (v/v) foetal bovine serum, 2 mM L-glutamine, HAT media supplement (50x Hybri-Max) TM. Media was filtered using a 0.2 µm Sartorius Sartolab V150 filter unit and stored at 4⁰C until use.

2.3.27 Preparation of F11 cell differentiation media:

DMEM, 15% (v/v) foetal bovine serum, 2 mM L-glutamine, 0.5 mM dibutyryl cyclic AMP (db-cAMP). Filter sterilised.

2.3.28 Loading buffer:

0.25% (w/v) bromophenol blue, 30% (v/v) glycerol and 60 mM EDTA pH 8.0

2.3.29 Ringer buffer:

160 mM Sodium chloride , 2.5 mM Potassium chloride, 5 mM Calcium chloride, 1 mM Magnesium chloride, 8 mM Glucose, 10 mM Hepes

2.3.30 High potassium buffer:

112.5 mM Sodium chloride, 50 mM Potassium chloride, 5 mM Calcium chloride, 1 mM Magnesium chloride, 8 mM Glucose, 10 mM Hepes

2.4 General methods

2.4.1 Membrane preparation for immunoblotting

Adult male Wistar rats and C57bl/6 mice supplied by Charles River Laboratories (Kent, UK) were euthanised by Schedule 1 methods under the Scientific Procedures Act (1986), either stunned or placed in a CO₂ chamber and 100% CO₂ was added at a flow rate of 20% of the chamber volume per minute. The mice were then decapitated. Following decapitation, spinal cords from the lumbar region and skin from the plantar hind surface of the hind paw were dissected and kept cool on ice. Using 200 µl of cold homogenisation buffer (50 mM Tris-HCl, pH 7.4 containing 5 mM EDTA, 5 mM EGTA), spinal cord tissue was homogenised in a Dounce glass/glass homogeniser. Membrane debris was pelleted by centrifugation of the homogenate at 1,200 x g, 4°C for 10 minutes.

The supernatant was transferred to a clean JA20 centrifugation tube; the volume was made up to 10 ml with homogenisation buffer + sucrose and was spun at 20,000 x g, 4°C for 30 minutes. The supernatant was discarded and the pellet re-suspended in 5 ml of homogenisation buffer, for every gram of starting material. The homogenate was stored in 100 µl aliquots at -20°C. The remaining pellet was resuspended in cold homogenisation buffer and the latter two steps repeated. The spinal cord preparation was then aliquoted into 100µl fractions and frozen at -20°C. The protein concentration was determined by a Lowry assay (Lowry *et al.* 1951).

2.4.2 Lowry assay for protein determination

The protein concentration was determined using the method of Lowry *et al.* (1951) employing bovine serum albumin (BSA) as the standard protein. All samples were assayed in triplicate. A stock solution of BSA (10 mg/ml) was serially diluted in water, to give a range of standard BSA concentrations ranging from 0-100 µg/ml. Lowry reagent **A** [2% (w/v) sodium carbonate, 0.1 M sodium hydroxide, 0.5% (w/v) lauryl sulphate (SDS)], Lowry reagent **B** [2% (w/v) sodium potassium tartrate] and Lowry reagent **C** [1% (w/v) copper sulphate] were mixed in a volume ratio of **A** (100): **B** (1):

C (1) respectively to form reagent D. To both the BSA standards and the unknown protein samples (50 μ l) 0.5 ml of reagent D was added to each sample and vortexed before incubation at room temperature for 10 minutes. On the addition of 50 μ l of Folin-Ciocalteu phenol reagent (1 M, 1:1 mix of Folin reagent and water) each sample was mixed and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of 500 μ l of water. The absorbance of each tube was determined and the optical density (O.D). At $\lambda = 750$ nm, was determined for each sample using a Jenway Genova Spectrophotometer.

A calibration curve was constructed of O.D. at $\lambda = 750$ nm for the BSA samples. This was then used to determine the unknown protein concentration.

2.4.3 Preparation of resolving gel

The resolving gel (10%) was prepared by mixing water (6 ml) with resolving gel buffer (3 ml), TEMED (6 μ l), stock acrylamide (3 ml) and 10% (w/v) ammonium peroxodisulphate (APS) (60 μ l). The polyacrylamide solution was immediately poured into a Hoefer SE 245 dual gel caster, using 2 gel plates of 10 x 8 cm and spacers of 1 mm width. Saturated butanol (10% w/v) and water solution (100 μ l) was added over the top of each gel. The gels were covered with parafilm and were allowed to polymerise for 60 minutes at room temperature. Gels were individually wrapped in tissue and stored in electrode buffer at 4°C until use.

2.4.4 SDS-Polyacrylamide Gel Electrophoresis

The resolving mini-slab gel was clamped into a Hoefer mini-vertical gel electrophoresis unit SE260. The stacking gel was prepared by mixing water (2.3 ml) with stock acrylamide (650 μ l), stacking gel buffer (1 ml) and TEMED (5 μ l) and 10% (w/v) ammonium persulphate (80 μ l) was added to the stacking gel and this was immediately poured into the mini-slab gel above the resolving gel. A welled comb was inserted into the stacking gel and removed after the polymerisation of the stacking gel and the wells were washed with buffer. Approximately 300 ml of electrode buffer was poured into the wells and into the base of the electrophoresis

unit. 15 µl of protein samples and pre-stained standards (protein molecular weight range of 180 - 26 kDa, 15 µl) were loaded to the wells of the stacking gel using a Hamilton syringe. Electrophoresis was carried out at 180 V, 10 mA (per gel) and was increased by 5 mA (per gel) once the samples reached the resolving gel. The gel was run for approximately 2 hours until the appropriate pre-stained molecular weight marker (25 kDa) reached the bottom of the gel.

2.4.5 Immunoblotting

After SDS-PAGE, the proteins from the gels were transferred to nitro cellulose membranes. A transfer cassette sandwich was constructed with the following order of components which had been pre-equilibrated in transfer buffer; sponge, two sheets of blotting paper and nitrocellulose membrane. The SDS-PAGE gel, two sheets of blotting paper and a final piece of sponge were added to the transfer cassette sandwich. On the addition of each component to the transfer cassette sandwich air bubbles were carefully removed by pressing each layer with a test tube. Proteins were transferred at a constant voltage of 50 V for 2.5 h using a Hoefer TE 22 tank transfer unit containing transfer buffer kept cool with ice and cold packs.

Following the transfer of the proteins, the nitrocellulose membrane was briefly rinsed with TBS and incubated with blocking buffer which was TBS, containing 5% (w/v) dried milk and 0.02% (v/v) Tween-20 (15 ml) for 1 h at room temperature with gentle shaking. After blocking of the non-specific antibody sites the nitrocellulose membranes were washed with ~ 10 ml of Tris Buffered Saline. The appropriate affinity-purified primary antibodies were diluted in incubation buffer of TBS, pH 7.4 containing 2.5% (w/v) dried milk to working concentrations (0.5-5 µg/ml). The nitrocellulose membranes were incubated with the diluted primary antibody solution (10 ml) overnight at 4⁰C with gentle shaking.

After incubation with the primary antibodies the nitrocellulose membranes were washed four times in wash buffer containing TBS with 2.5% (w/v) dried milk and 0.2% (v/v) Tween-20 (10 ml) at 10 minute intervals with gentle shaking at room

temperature. Nitrocellulose membranes were then incubated for 1 hour with gentle agitation with horseradish peroxidase (HRP) labelled secondary antibody, either anti-rabbit or anti-mouse depending on what primary antibody was raised in, at a dilution of 1/2000 in incubation buffer (10 ml). The unbound secondary antibody was removed by washing the membrane as described above. The membrane was then drained of excess buffer and briefly rinsed in TBS. Immunoreactive bands on the nitrocellulose membrane were developed by processing in a solution containing Tris-HCl pH 8.5 (1 M), luminol (250 mM), coumaric acid (90 mM) and hydrogen peroxide (30%, 64 μ l) for 1 minute at room temperature with gentle shaking. After removal of the reagents, the immunoblot was wrapped in cling film, placed in a film cassette and exposed to HyperfilmTM for various times (1-10 minutes). The film was then developed in KodakD-19 Developer until the immunoreactive bands were visible and fixed in Kodak Unfix for 5 minutes at room temperature.

2.4.6 Immunofluorescence analysis

Immunohistochemical analysis was carried out by conventional procedures as described previously by (Chazot et al., 2001). The lumbar spinal cord sections from healthy and pain models kindly provided by Pfizer (Freund's complete adjuvant model) were perfusion fixed with 4% (w/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The spinal cords were removed, post-fixed in paraformaldehyde overnight and then cryoprotected by incubation in 30% (w/v) sucrose in 0.1 M phosphate buffer, pH 7.4 at 4⁰C for 48 hours. The tissue was then frozen in isopentane at -80⁰ C for 1.5 minutes and horizontal sections (20 μ m) were cut on a cryostat. Skin from the plantar surface of the hind paw was processed similarly and 8 μ m sections were cut on the cryostat.

Free floating sections of the lumbar spinal cord were initially treated with 10% (v/v) methanol and 3% (v/v) hydrogen peroxide in 50 mM TBS, pH 7.4 for 30 minutes to quench endogenous peroxidase activity (Wendelboe and Bisgaard., 2009). The sections were then given 3 X 5 minute washes in 0.2% triton x-100 (v/v) TBS solution.

Sections were incubated in TBS, 0.2% (w/v) glycine to mop up residual unreacted aldehyde groups from the fixative. Non-specific binding sites in the tissue were blocked by incubating with foetal calf serum 2% (v/v) in TBS/triton x-100 0.2% (v/v) for 60 minutes at room temperature with gentle agitation. Sections were then incubated overnight at 4⁰C in anti-hH₄(374-390) antibody at a final concentration of 1 µg/ml in foetal calf serum/TBS 1% foetal calf serum, (v/v). After the overnight incubation, sections were given 3 X 5 minutes washes with 0.2% triton-x 100 and TBS, before 2 hour room temperature incubation in anti-rabbit Alexa Fluor 594. After the incubation period, the sections were given 3 X 5 minute washes with 0.2% triton-x 100 and TBS. In addition, a control was carried out where the primary antibody was left out, so as to rule out non-specific binding of the secondary antibody to the slice.

After immunostaining, all sections were mounted onto gelatin-coated slides, dried, dehydrated and cleared in xylene. Coverslips were applied with DPX-mount mounting medium. Sections were examined using a Zeiss Axioskop 2 equipped for epifluorescence.

2.4.7 Immunofluorescence for histamine H₄ receptor antibodies and Neuronal markers

Refer to the immunofluorescence protocol in section 2.4.6

After blocking of non-specific binding sites, the sections were then incubated overnight at 4⁰C in anti-hH₄ antibody at a final concentration of 1 µg/ml in foetal calf serum/TBS (1% serum, v/v) guinea pig anti-substance P (1:300 dilution) or anti-hH₄ at 1 µg/ml and mouse anti-calcitonin gene-related peptide (1:300 dilution). After the overnight incubation, sections were given 3 X 5 minutes washes 0.2% triton-x 100 and TBS, before a 2 hour room temperature incubation in anti-rabbit Alexa Fluor 594, anti-mouse Alexa Fluor 488 or anti-guinea pig Alexa Fluor 488 secondary antibodies. In addition, a control was carried out where the primary antibody was left out, so as to rule out non-specific binding of the secondary antibody to the slice.

2.4.8 Poly-D-Lysine coating of cover-slips

5 mg of poly-D-lysine was dissolved in 50 ml of tissue culture grade water and mixed vigorously until the contents had dissolved. 1 ml per 25 cm² of dissolved poly-D-lysine was used to coat the cover slips and the plate was rocked gently to ensure uniform coating of culture surface and left to dry overnight under UV light in the tissue culture hood. After overnight drying, the coverslips were rinsed with PBS. Media and cells were introduced to the poly-D-lysine coated cover slips and incubated at 37⁰C, 5% CO₂.

CHAPTER 3

The histamine H₄ receptor is expressed on sensory neurones

3.1 Objectives

The aim of this chapter was to determine the expression of the histamine H₄R on sensory nerve fibres. The next aim was to perform a series of pharmacological experiments to determine the functional expression of this receptor on dorsal root ganglion neurons in order to define a new model system.

3.2 Introduction

Histamine is the best known endogenous agent implicated for the induction of itch and also produces pain upon release from mast cells. Itch or 'pruritus' is defined as an unpleasant cutaneous sensation associated with the desire to scratch (Steinhoff et al., 2006). Chronic intense itching can lead to the development of skin lesions and release of inflammatory mediators by epithelial, endothelial or immune cells that induce or aggravate pruritus, resulting in reinforcement of scratching. This is referred to as the "itch-scratch cycle" (Figure 3.1)

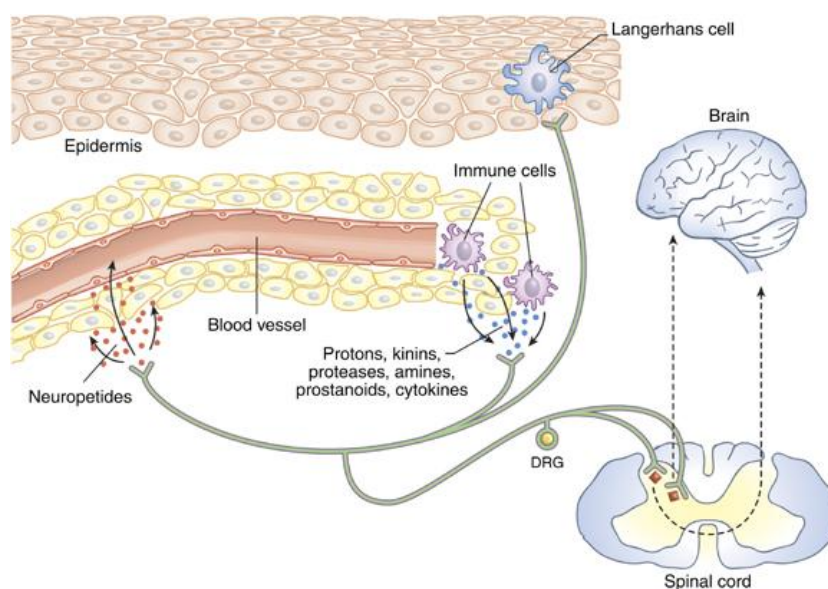


Figure 3.1 The itch scratch cycle (Steinhoff et al., 2006).

Exogenous and endogenous factors (released by endothelial cells, epithelial cells, and immune cells) activate signalling pathways from the periphery via the dorsal root ganglia (DRG) and spinal cord, to the brain. Activation of specific areas in the brain results in the perception of itch, which produces a scratching response.

Histamine receptor involvement in itch

Histamine acts via four G-protein coupled receptors H_1 -, H_2 -, H_3 - and H_4 receptors. The H_1 Rs are involved in histamine-induced itch, as agonists for these receptors provoke itch in mice, and antagonism of these receptors prevents histamine and allergen-induced itch (Rossbach et al., 2009, Bell et al., 2004). Although traditional H_1 R antagonists are widely used and moderately effective in the treatment of histamine-induced itch, they are not clinically effective in conditions such as atopic eczema. The H_2 R is only partially involved in the mediation of itch as the H_2 R antagonist cimetidine, did not significantly reduce histamine-induced itch (Bell et al., 2004). The H_3 R antagonists thioperamide and clobenpropit dose-dependently induced scratching, but these compounds have been shown to possess affinity for the H_4 R (Tiligada et al., 2009). According to Bell *et al.* (2004), intradermally administered thioperamide induced itch in mice, while systemic administration dose-dependently reduced histamine or clobenpropit-induced scratching behaviour (Bell et al., 2004).

The H_4 R agonist 4-Methylhistamine dose-dependently induced scratching in wild-type and mast cell deficient mice, an effect that was inhibited by administration of the specific H_4 R antagonist JNJ 7777120 (Dunford et al., 2007). Several studies have presented the inhibition and amelioration of pruritus using H_4 R antagonists (Yamaura et al., 2009, Suwa et al., 2011, Dunford et al., 2007). Based on these findings, the presence of the H_4 R on sensory neurones was proposed and H_4 R expression was detected in the peripheral nervous tissue. High H_4 R mRNA was detected the human and rat DRG (Strakhova et al., 2009). DRG neurons contain the cell bodies of sensory afferents and this further supports the hypothesis of the potential involvement of the H_4 R in the neural transmission of itch.

In humans, histamine-induced itch is transmitted by specific mechanosensitive C-fibers, which are preferentially activated by pruritogens like histamine (Schmelz et al., 1997). Recently, the functional expression of the H_4 R on sensory neurones was reported. Activation of the H_4 R with 4-Methylhistamine induced a strong intracellular calcium increase comparable to that induced by capsaicin, in subset of

capsaicin-sensitive DRG sensory neurones. This effect was absent in transient potential receptor vanilloid knockout (TPRV $-/-$) mice (Rossbach et al., 2011). This further implies that the H₄R is a promising target for the management of chronic pruritus.

Histamine plays complex role in the modulation of pain as it is an established mediator of acute and chronic inflammatory conditions (Thurmond et al., 2008b). The close proximity of mast cells, microvessels and sensory fibres may permit the contribution of histamine and other mast cell products such as prostaglandins to the pain sensation. Central activation of the H₁R induces antinociception, where as histamine was demonstrated to stimulate nociceptive fibres through H₁R activation in rodents (Parada et al., 2001). Activation of peripheral and central H₃R has been shown to inhibit formalin-induced nociception (Cannon et al., 2007b, Cannon, 2003). Recent studies also suggest that the H₄R could be involved in the acute carrageenan-induced inflammatory pain (Coruzzi et al., 2007) and neuropathic pain (Hsieh et al., 2010b).

In light of this, we set out to localise the expression of the histamine H₄R on peripheral sensory afferent fibres in the skin, DRG and spinal cord using immunohistochemical methods. During itch and nociception, nerve endings release neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) (Mogil et al., 2005), which contribute to and aggravate the condition by sensitising peripheral afferents. Therefore, we analysed expression of histamine H₄R in relation to sensory fibres that express these neuropeptides. Another aim was to develop a functional expression model of the H₄R in primary rodent DRG neurons, using calcium signalling.

3.3 Methods

3.3.1 Tissue preparation

Following cervical dislocation, spinal cord, dorsal root ganglia and skin from four adult male Wistar rats were dissected and stored in 4% formalin before snap freezing, in preparation for cryostat sectioning. Immunohistochemical analysis was then performed on skin from the plantar surface of the hind paw, lumbar spinal cord and dorsal root ganglia using the panel of selective antibodies. Controls were carried out in the absence of primary antibodies.

3.3.2 Immunofluorescence for histamine H₄R and substance P/CGRP

Refer to section 2.4.7

3.3.3 Isolation of DRG neurons

Three 14 day old male Wistar rats were deeply anaesthetised with isoflurane and then decapitated. The spinal column was opened and 10-12 DRG along the whole vertebral column were dissected. Isolated ganglia were enzymatically digested in Ca²⁺ and Mg²⁺ free filter sterilised Hanks Balanced Salt solution (HBSS) supplemented with collagenase crude type 1A (1 mg/ml) and dispase (10 mg/ml), at 37°C for 20-30 minutes. Neurons were dissociated by trituration using fire-polished Pasteur pipettes of decreasing tip pore size. Cells were washed in 10 ml of fresh growth media, DMEM GlutaMax I supplemented with 10% foetal calf serum, penicillin 50 U/ml and streptomycin 50 µg/ml. Neurons were then collected by centrifugation at 800 rpm, 4°C for 5 minutes and the supernatant discarded. The pellet was then washed with fresh growth media and gently triturated using a 1 ml Gilson pipette. Neurons were then collected by centrifugation at 800 rpm, 4°C for 5 minutes and the pellet resuspended in 800 µl of warmed growth media. The neurons were plated onto coverslips at 100 µl per 10 mm coverslip in a 24 well plate. The plate was then incubated at 37°C for 2-3 hours to allow the cells to adhere to the coverslips, after which the wells were flooded with 1 ml of pre-warmed DMEM media. The cells were cultured for 2-3 days at 37°C in 5% CO₂.

3.3.4 Calcium²⁺ imaging of DRG neurons stimulated with the H₄R agonist, VUF 8430

Changes in the intracellular calcium concentration $[Ca^{2+}]_{free}$ of single DRG cells were measured by digital microscopy, using a Nikon eclipse TE-2000E connected to equipment for recording of single cells.

Coverslips containing the DRG neurons were loaded with Fluo-4 AM (fluorescent cell permeant acetoxymethyl ester) in DMSO (1 μ l/ μ l), in Ringer solution (artificial cerebral spinal fluid) containing 10% Pluronic-F127 solution (0.5 μ l/ml) and incubated for 40 minutes at 37°C in 5% CO₂. For imaging, the coverslip was placed in a chamber on the stage of the microscope and constantly perfused with Ringer solution at 35°C. The field of cells was monitored by observing epifluorescence after excitation at the 488 nm and images were acquired every 1 second, by programming the NiS Elements™ software to automatically do so. Neurons were initially identified at the beginning of each experiment by exposure to 50 mM KCl solution. Neurons were initially exposed to control (Ringer solution), followed by 50 mM KCl, 100 μ M Histamine, 1 μ M VUF 8430 (specific H₄R agonist, kind gift of Prof Rob Leurs, Amsterdam), 1 μ M Capsaicin and 10 μ M JNJ 7777120 (specific H₄R antagonist). 1 ml of each stimulus was applied to the coverslip and the cells were washed with fresh buffer for at least 2 minutes after application of each stimulus, to allow the cells to recover the cells prior to addition of the next stimulus.

3.4 Results

3.4.1 IHC showing substance P and CGRP labelling of peptidergic C fibres in the dorsal horn of the rat lumbar spinal cord

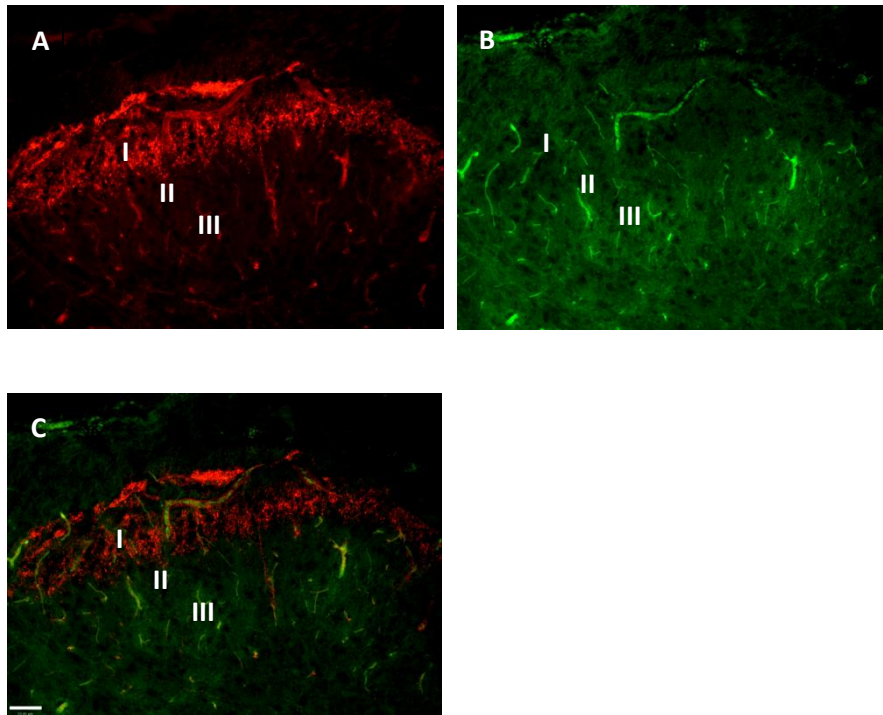


Figure 3.1 Immunohistochemistry of rat lumbar spinal cords probed with **A** anti-Calcitonin gene-related peptide (red) **B** anti-substance P (green). **C** represents a double labeled merged image of anti-CGRP and anti-substance P immunoreactivity in the rat lumbar spinal cord. Immunostaining of rat 20 μm transverse lumbar spinal cord slices (X20). Scale bar = 10 μm and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.1 shows IHC labelling of sections of the dorsal horn from the lumbar region of the spinal cord. CGRP is expressed in lamina I and outer lamina II. Substance P is expressed in lamina II of the dorsal horn. There is no evidence of overlap between the two neuropeptides.

3.4.2 IHC showing substance P and CGRP labelling of cell bodies in the dorsal root ganglia of the rat

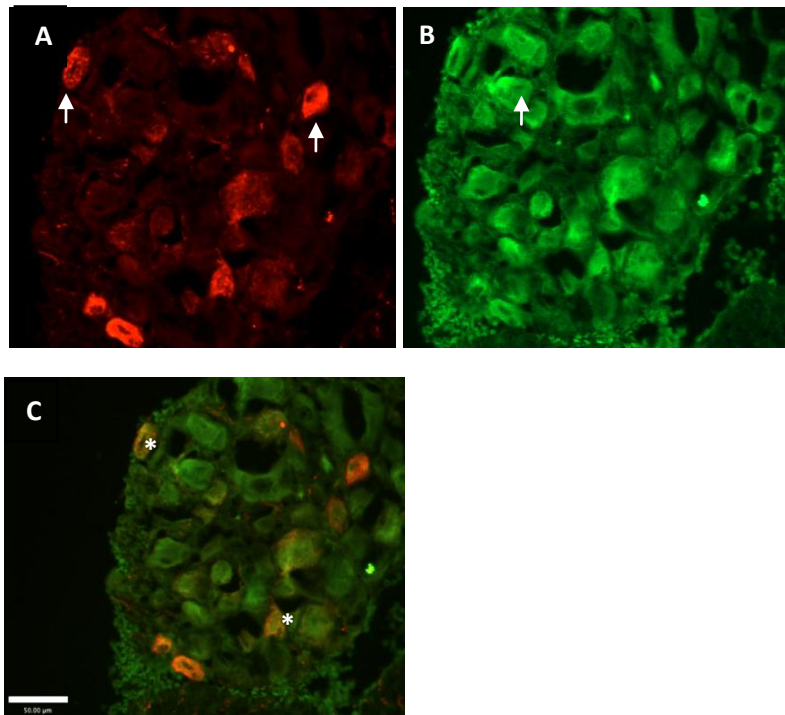


Figure 3.2 Immunohistochemistry of rat L4-L5 DRG cells probed with **A** anti-calcitonin gene-related peptide (red) **B** anti-substance P (green). **C** represents a double labeled merged image of anti-CGRP and anti-substance P immunoreactivity in the rat DRG. Immunostaining of 6 μm transverse rat DRG slices (X20). Scale bar = 50 μm and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.3 shows IHC labelling of the cells the dorsal root ganglia. CGRP is expressed on a subset of DRG neurons, which are putative A δ fibres and substance P on a subset of putative C-fibres. There is no evidence of overlap in expression of the neuropeptides.

3.4.3 IHC showing substance P and Isolectin B4 labelling of peptidergic and non-peptidergic C fibres in the dorsal horn of the rat lumbar spinal cord

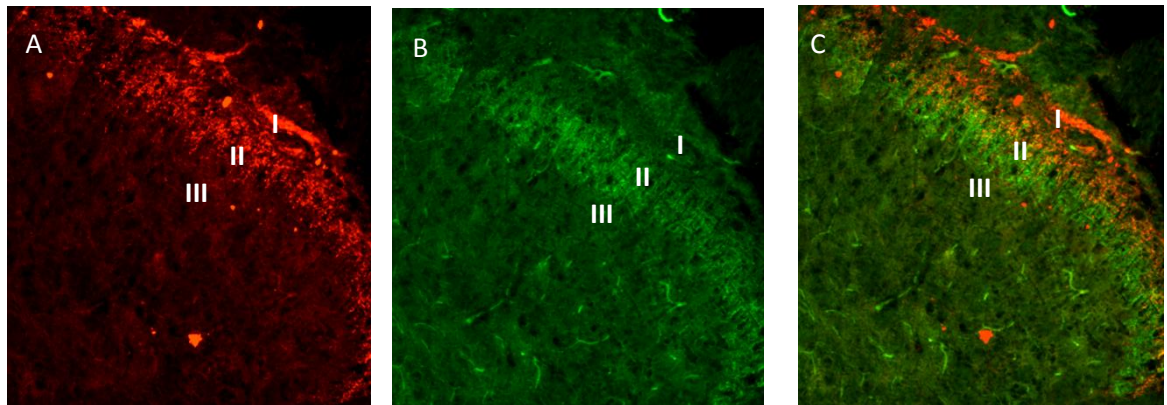


Figure 3.3 Immunohistochemistry of rat lumbar spinal cords probed with **A** anti-substance P (red) **B** anti-IB4 (green). **C** represents a double labeled merged image of anti-substance P and anti-IB4 immunoreactivity in the rat lumbar spinal cord. Immunostaining of rat 20 μm transverse lumbar spinal cord slices (X20). Scale bar = 10 μm and represents all images shown. All immunohistochemistry data shown are representative images from at 3 experiments.

Figure 3.3 shows the IHC of the peptidergic putative C-fbres in dorsal horn of the spinal cord with anti-substance P, and the non-peptidergic C-fbres with Isolectin B4. Substance P is expressed on neurons terminating in lamina I and outer II, where as Isolectin B4 is expressed on neurons terminating within inner lamina II.

3.4.4 IHC showing substance P and Isolectin B4 labelling of cell bodies in the dorsal root ganglia of the rat

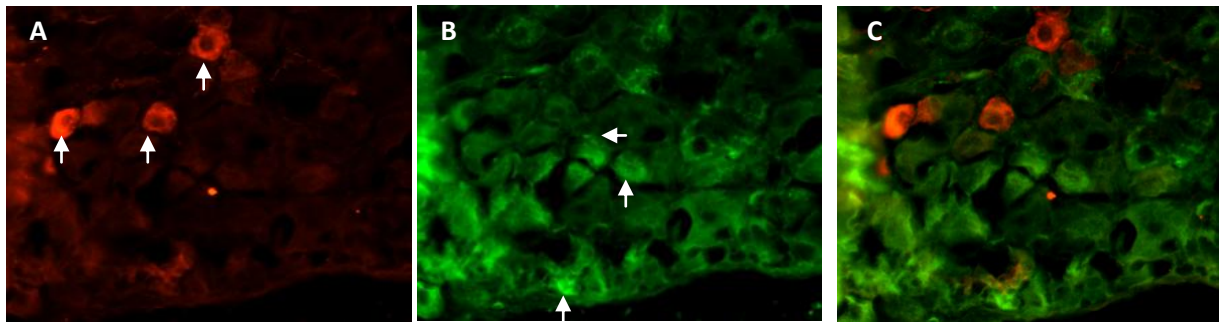


Figure 3.4 Immunohistochemistry of rat L4-L5 DRG cells probed with **A** anti-substance P (red) **B** anti-IB4 (green). **C** represents a double labeled merged image of anti-substance P and anti-IB4 immunoreactivity in the rat DRG. Immunostaining of 6 μ m transverse rat DRG slices (X20). Scale bar = 50 μ m and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.4 shows the IHC of the peptidergic and non-peptidergic putative C-fibre neurons in the dorsal root ganglia. There is no observed overlap in expression of the substance P-expressing and Isolectin B4- expressing neurons.

3.4.5 IHC showing H₄R and Substance P labelling in the skin from the plantar surface of the hind paw of the rat

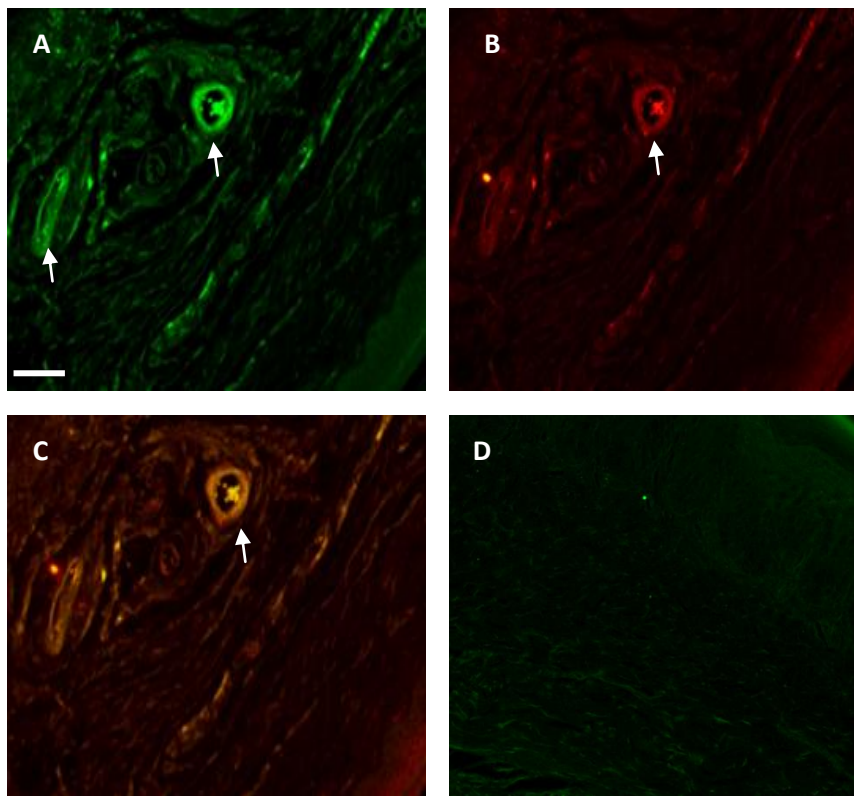


Figure 3.5 immunohistochemistry of skin from the plantar hind surface of the hind paw probed with **A**, anti-H₄R antibody (green), **B** anti-substance P (red), **C** represents a double labeled merged image of A and B. D is the control experiment without the primary or secondary antibody. Immunostaining of skin from the plantar surface of the right hind paw of the rat, 8 μ m transverse skin slices (X20). Scale bar = 50 μ m and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.5 shows IHC of skin from the plantar surface of the hind paw of the rat. The H₄R antibody appears to label the substance P-positive C-fibres, which innervate the blood vessels (arrows). This represents the co-expression of the histamine H₄R on substance P C-fibres.

3.4.6 IHC showing H₄R and Substance P labelling in the dorsal root ganglia of the rat

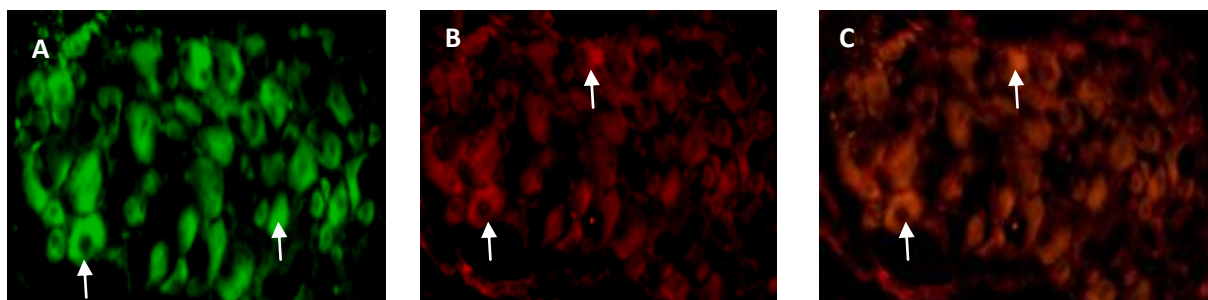


Figure 3.6 Immunohistochemistry of rat L4-L5 DRG cells probed with **A** anti-H₄R antibody (green), **B** anti-substance P (red), **C** represents a double labeled merged image of anti-substance P and anti- H₄R immunoreactivity in the rat DRG. Immunostaining of 6 μ m transverse rat DRG slices (X20). Scale bar = 50 μ m and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.6 shows the IHC labelling of neurons of the DRG. The H₄R appears to be expressed on a subset of neurons which are positive for substance P. This represents the co-expression of the histamine H₄R on a subset of substance P-expressing putative C-fibres.

3.4.6.1 Quantification of DRG neurones that express H₄R and substance P immunoreactivity (IR)

Neuron size	Percentage of neurons (%)		
	Substance P-IR	H ₄ R-IR	Sub P/H ₄ R-IR
Small (<25 μ m)	7.7 (20/259)	10 (26/259)	4.6 (12/259)
Medium (25-50 μ m)	15 (39/259)	10.8 (28/259)	6.5 (17/259)
Large (>50 μ m)	13.5 (35/259)	10.4 (27/259)	16.6 (43/259)

Table 3.1 represents shows the percentage of DRG sensory neurons positive for substance P expression, H₄R and double labelled substance P-H₄R immunoreactivity. The neurons were counted and the diameters measured using Image J software.

3.4.7 IHC showing histamine H₄R and substance P labelling of C fibres in the dorsal horn of the rat lumbar spinal cord

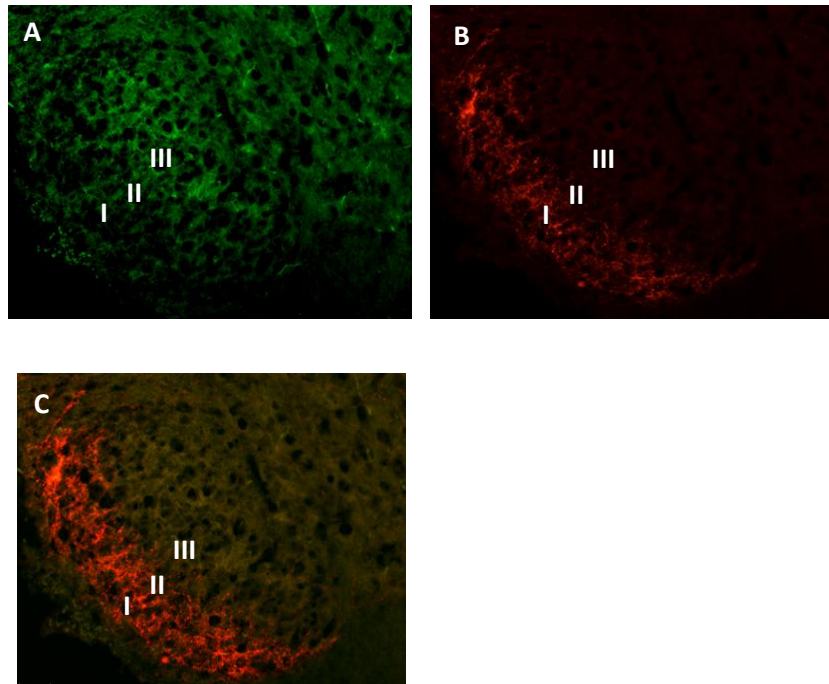


Figure 3.7 Immunohistochemistry of rat lumbar spinal cords probed with **A** H₄R antibody (green), **B** anti-substance P (red) **C** represents a double labeled merged image of anti-substance P and anti-IB4 immunoreactivity in the rat lumbar spinal cord. Immunostaining of rat 20 μm transverse lumbar spinal cord slices (X20). Scale bar = 10 μm and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.7 shows IHC of the dorsal horn of the lumbar region of the spinal cord. There is some labelling of the histamine H₄R on neurons terminating in the dorsal horn. Substance P is expressed on neurons terminating within lamina I and II of the dorsal horn. There is no observed co-expression of the H₄R and substance P on the neurons within the spinal cord.

3.4.8 IHC showing H₄R and CGRP labelling in the skin from the plantar surface of the hind paw of the rat

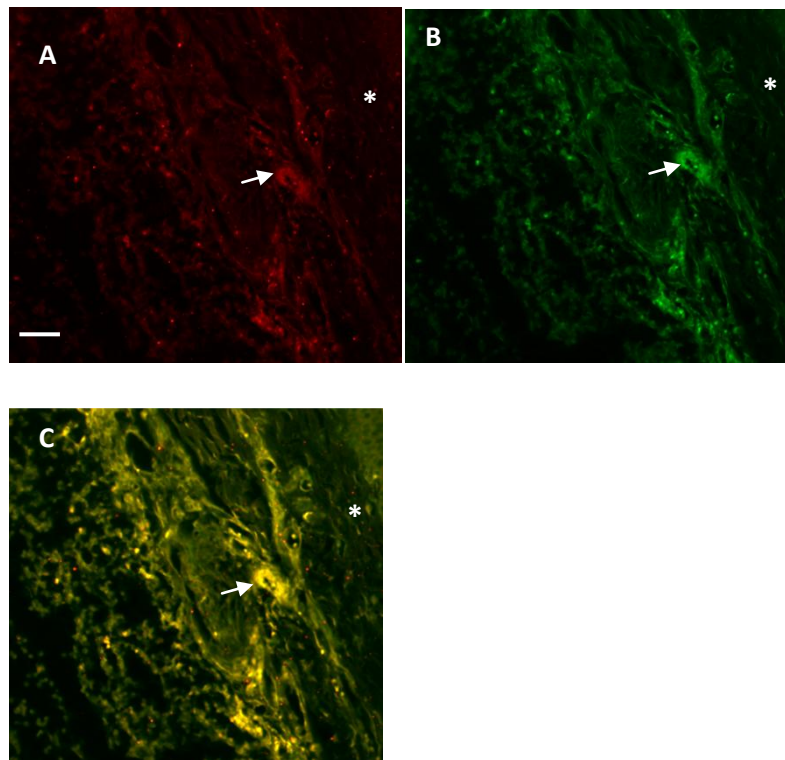


Figure 3.8 immunohistochemistry of skin from the plantar hind surface of the hind paw probed with **A**, anti-H₄R antibody (green), **B** CGRP (red), **C** represents a double labeled merged image of A and B, and yellow indicated double labelling. Immunostaining of skin from the plantar surface of the right hind paw of the rat, 8 μ m transverse skin slices (X20). Scale bar = 50 μ m and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.8 shows IHC labelling of skin from the plantar surface of the hind paw of the rat. There is expression of the histamine H₄R on the fibres innervating the blood vessels (arrow) as well putative A δ -fibres innervating the dermis (asterix). There appears to be co-expression of the H₄R on CGRP expressing fibres.

3.4.9 IHC showing H₄R and CGRP labelling in the dorsal root ganglia of the rat

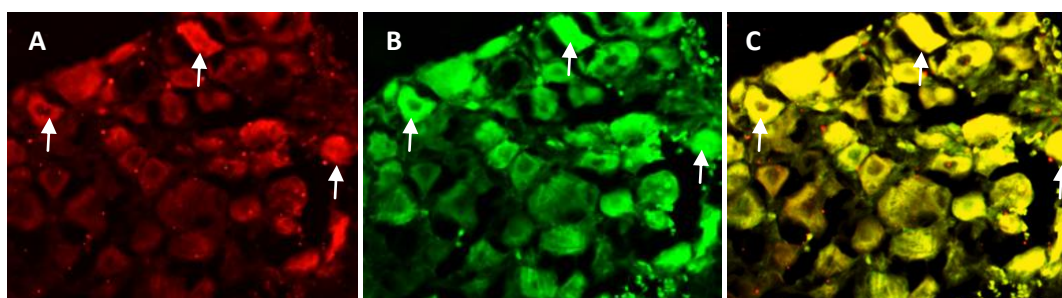


Figure 3.9 Immunohistochemistry of rat L4-L5 DRG cells probed with **A** anti-H₄R antibody (red), **B** anti-CGRP (green), **C** represents a double labeled merged image of anti-CGRP and anti-H₄R immunoreactivity in the rat DRG. Immunostaining of 6 μ m transverse rat DRG slices (X20). Scale bar = 50 μ m and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.9 represents IHC labelling of neurons from the DRG. The anti-H₄R antibody has labelled a subset of DRG neurons. CGRP has labelled a subset of putative A δ -fibres. There appears to be a subset of double labelled neurons, putative A δ -fibres within the DRG.

3.4.9.1 Quantification of DRG neurones that express H₄R and calcitonin gene-related peptide immunoreactivity (IR)

Neuron size	Percentage of neurons (%)		
	CGRP-IR	H ₄ R-IR	CGRP/H4R-IR
Small (<25 μ m)	23.2 (57/246)	12.2 (30/246)	17.5 (43/246)
Medium (25-50 μ m)	10.9 (27/246)	17.1 (42/246)	10.9 (27/246)
Large (>50 μ m)	1.2 (3/246)	3.7 (9/246)	0

Table 3.2 shows the percentage of DRG sensory neurons positive for CGRP expression, H₄R and double labelled CGRP-H₄R immunoreactivity. The neurons were counted and the diameters measured using Image J software.

3.4.10 IHC showing histamine H₄R and CGRP labelling of C fibres in the dorsal horn of the rat lumbar spinal cord

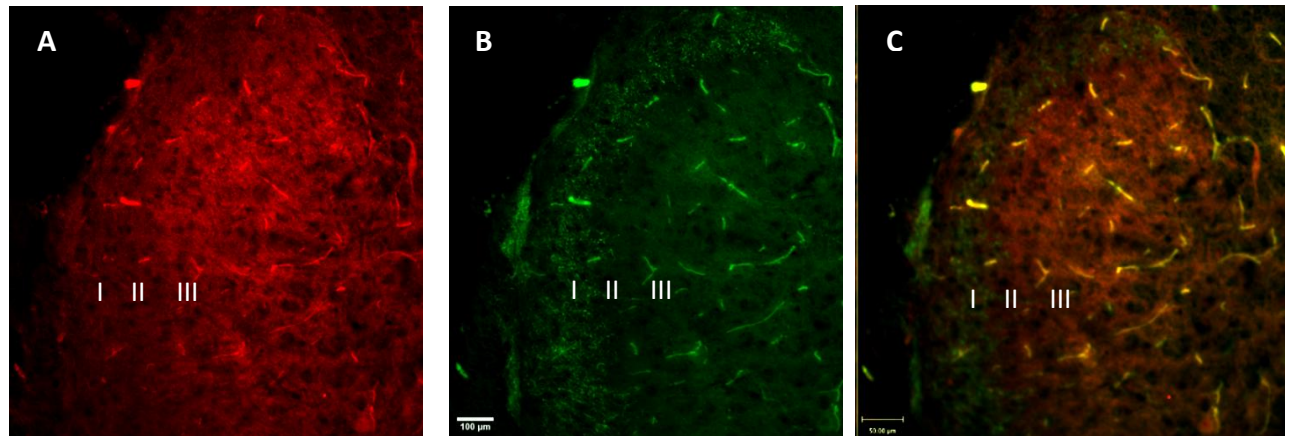


Figure 3.10 Immunohistochemistry of rat lumbar spinal cords probed with **A** H₄R antibody (red), **B** anti-CGRP (green) **C** represents a double labeled merged image of anti-CGRP and anti-H₄R immunoreactivity in the superficial layers of the rat lumbar spinal cord, yellow represent double-labelling. Immunostaining of rat 20 μm transverse lumbar spinal cord slices (X20). Scale bar = 100 μm and represents all images shown. All immunohistochemistry data shown are representative images from 3 experiments.

Figure 3.10 shows IHC labelling of the dorsal horn of lumbar spinal cord. There appears to be co-expression of H₄R and CGRP on some putative Aδ fibres terminating within the deeper layers of the lumbar spinal cord

3.4.11 Functional expression of the histamine H₄ receptor in the dorsal root ganglia of rat using the selective H₄ receptor agonist VUF 843

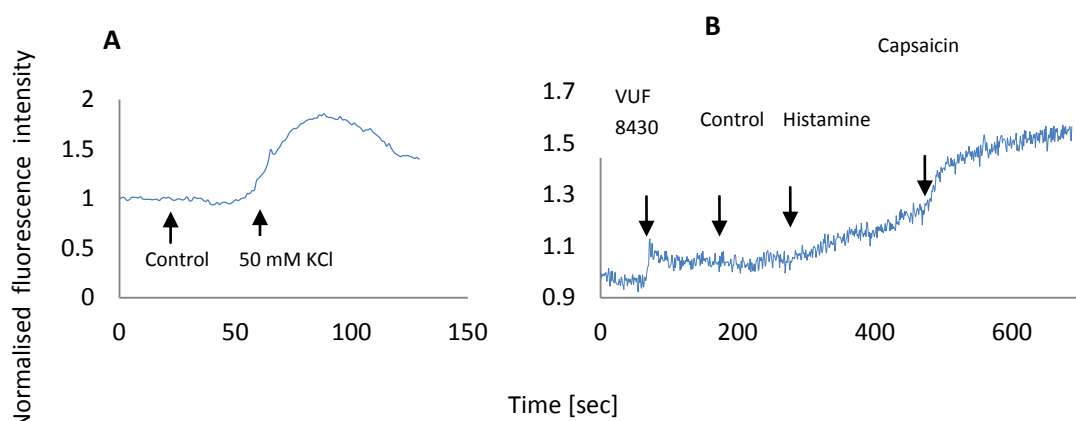


Figure 3.11 Changes in normalised fluorescent intensity of DRG neurons in response to control, 50mM KCl, 1 μ M VUF 8430, 100 μ M histamine and 1 μ M capsaicin induced Ca^{2+} release. Following KCl stimulation, the cells were washed with fresh buffer for at least 2 minutes to recover the cells, prior to the addition of the next stimulus. The fluorescent intensity of each cell was normalised for graphical representation.

Figure 3.11 [A] Neurons were identified as cells responsive to 50 mM KCl. [B] The capsaicin sensitive cells were responsive to histamine and the H₄R agonist, VUF 8430. There is an increase in the observed normalised fluorescent intensity following administration of VUF 8430, histamine and capsaicin. The normalised fluorescent intensity was used to measure H₄R activity through intracellular calcium mobilisation from intracellular stores. This is consistent with an increase in the release of intracellular calcium from the DRG neurons.

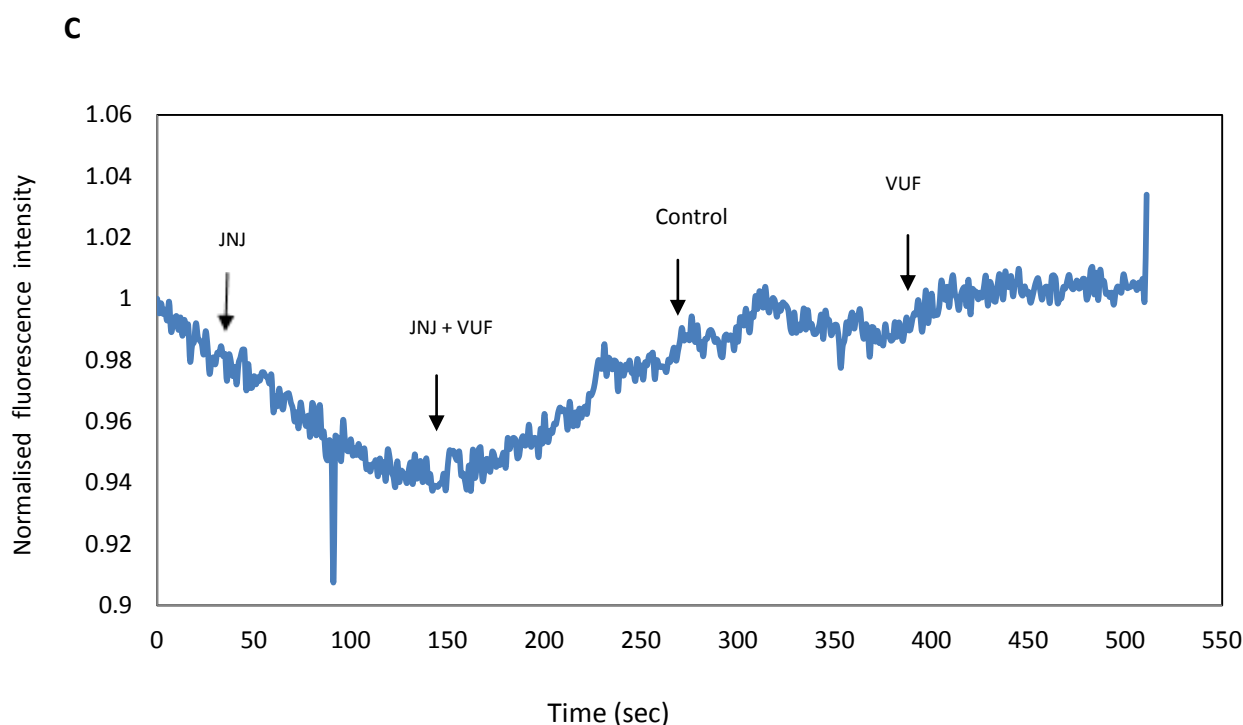
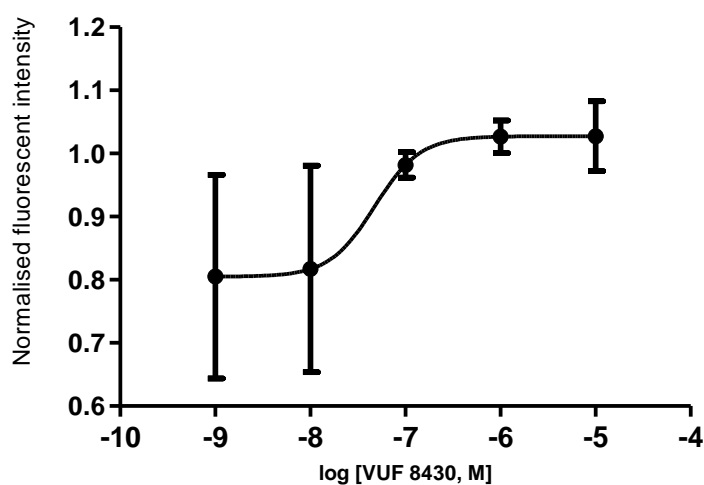


Figure 3.12 Neurons were preanalysed with KCl for 2 minutes and then washed for 5 minutes. Cells were then continuously washed with control buffer before drug application. 10 μ M JNJ 7777120 was applied to the cells for 2 minutes followed by solution containing 1 μ M VUF 8430 and 10 μ M JNJ 7777120. Cells were washed for 2 minutes with the control solution before the addition of 10 μ M JNJ 7777120. The graph shows the normalised fluorescence induced by Ca^{2+} release from 100 mM KCl responsive cells, from 11 experiments.

Figure 3.12 shows that following administration of the selective H_4R antagonist JNJ 7777120, there is a reduction in the normalised fluorescence intensity observed in the cells. Addition of the solution containing the agonist produces an apparent increase of the normalised fluorescent intensity of the neurons. However, this effect was surmounted through application of 1 μ M VUF 8430, shown by the increase in the fluorescent intensity.

D Dose-response relationship between VUF 8430 concentration and the normalised calcium intensity



3.4.13 Neurons were detected by KCl stimulation for 2 minutes and washed for 5 minutes before VUF 8430 application for 3 minutes. The concentrations used ranged from VUF 8430 1×10^{-9} M to 1×10^{-5} M. Each concentration was repeated 3 times. pEC_{50} value = $7.3 \pm S.E.M.$

Figure 3.4.13 represents functional activity of the selective H_4R agonist VUF 8430 at the rat H_4R in the dorsal root ganglia neurons. The normalised fluorescent intensity was used to measure H_4R activity through intracellular calcium mobilisation from intracellular stores.

VUF 8430 acted as a full agonist at the rat H_4R , measured by the normalised fluorescent intensity and displayed a pEC_{50} value of 7.3 ($n=3$) at the rat H_4R .

3.5 Discussion

This chapter describes the immunohistochemical localisation of the histamine H₄R on putative C- and A δ nociceptive fibres. The H₄R is the most recently identified member of the histamine GPCR family, abundantly expressed on haematopoietic cells and its role in eosinophils, mast dendritic and T cell function points to a role in various allergic and inflammatory functions (Barnard et al., 2008, Dunford et al., 2006, Simon et al., 2010, Hofstra et al., 2003). In our attempts to determine the expression of H₄R on sensory neurones, antibodies were used to investigate the associations between the histamine H₄R, calcitonin gene-related peptide (CGRP) and substance P in skin, dorsal root ganglia neurons and the dorsal horn of the lumbar spinal cord of the adult male rat.

Histamine and other pruritogens are released from cutaneous mast cells which are located close to sensory fibres in the skin. Microdialysis experiments in human skin revealed that trauma such as insertion of a needle into the skin, can lead to a transient histamine release (Anderson et al., 1992). Histamine can excite a subpopulation of C-fibres nociceptors and a subpopulation of these has been shown to be insensitive to mechanical and heat stimuli but not histamine (Schmelz et al., 1997). Sensory receptors function as the first component in the ascending sensory pathway and respond to nerve growth factors as well as neuropeptides like substance P and calcitonin gene-related peptide. Immunohistochemistry was used to localise the distribution of substance P and our results are consistent with previously published data which shows that this neuropeptides is prominently expressed in small sensory primary C fibre afferents involved in the modulation of pain-related information in the dorsal horn of the spinal cord. The central terminations of substance P-containing neurons occur mainly in lamina I and outer lamina II (Mcleod et al., 1998). On the other hand, CGRP is expressed in small and intermediate sized lightly myelinated putative-A δ neurons in lamina I and II, also involved in nociception and the transmission of pain. This is consistent with our immunohistochemical findings.

3.5.1 H₄R expression on putative C- and A δ - sensory afferent neurons in skin from the plantar surface of the hind paw

Substance P-containing nerve endings originating from the DRG have been demonstrated immunohistochemically in nerves supplying small dermal vessels (O'Flynn et al., 1989) and free nerve endings and dermal papillae (Deguchi et al., 1989) in the epidermis. These are postulated to be nociceptors initiating pain transmission. Our data show the expression of the H₄R on substance P-positive nerve fibres innervating the dermis as well as surrounding the blood vessels in the dermis of the skin. Substance P has been proposed to be the main mediator of nociception and neurogenic inflammation through the initiation of plasma extravasation and vasodilatation by action on arterioles, after release by sensory C-fibres. NK1 antagonists have been shown to reduce hyperalgesia (Cahill andCoderre, 2002) and the symptoms of neurogenic inflammation (Moussaoui et al., 1993).

CGRP has been shown to exert vasodilative effects in the skin, where fmol-pmol amounts have produced prolonged increased blood flow (Brain et al., 1986). It has also been suggested that this neuropeptide may magnify the extravasation induced by mediators of increased vascular permeability, such as tachykinins (Gamse and Saria, 1985). Our IHC data shows the co-expression of the H₄R on fibres innervating the blood vessels in the skin, and fibres innervating the dermis. A study by Dunford *et al.* (2007) showed that the H₄R antagonist JNJ 7777120 was superior to the commonly used H₁R antagonist, diphenhydramine in the attenuation of experimental pruritus and lacked the sedative effects. The observation that the histamine-induced itch and the inhibitory effects of the H₄R antagonist were unaltered in mast cell-deficient mice (which have few or no mast cells in skin), indicated that the H₄R on mast cells was not required for initiation of H₄R-mediated pruritus. Substance P appears to be involved in the mediation of histamine-induced itch as scratching behaviour induced by the H₄R agonist, clobenpropit was reduced in mice treated with the neurokinin 1 (NK₁) antagonist spantide (Hossen et al., 2006). Similarly, substance P-induced itch was attenuated in mice pre-treated with the H₄R antagonist JNJ777120 (Yamaura et al., 2009). The anti-allergic effects of H₄R antagonists were evaluated in other models of dermatitis in mice. Rossbach *et al.*

(2009) showed that JNJ 7777120 pre-treatment significantly inhibited hapten-induced scratching but was unable to reduce the allergic inflammatory response, suggesting a non-immune cell acting site for the drug. The observation of the H₄R on substance P and CGRP positive neurons in the rat skin supports the hypothesis of a peripheral acting site for the H₄R antagonists which exert their effects by reducing inflammation (Hsieh et al., 2010a) and hapten-induced scratching in rodent models of pruritus (Dunford et al., 2007).

3.5.2 Expression of the H₄R in the dorsal root ganglia and spinal cord

Immunohistochemical detection revealed that the H₄R are expressed on the soma of sensory neurons, with more intense staining of the small and medium diameter sized neurons. In the lumbar spinal cord, expression was predominant in the superficial layers, lamina I and II, where the immunoreactivity pattern suggests possible localisation with the terminals of primary sensory afferent neurons from the DRG. In the rat, H₄R mRNA expression appears to be strongest in the nervous system with the highest expression detected in the DRG (Strakhova et al., 2009) by RT-PCR, in contrast to the human, where the strongest mRNA expression was observed in the spinal cord, cortex and cerebellum (Strakhova et al., 2009). The existence of the H₄R on peripheral afferent nerve terminals which mediate itch has been previously postulated (Dunford et al., 2007); therefore the location of the H₄R on soma of sensory neurons that express substance P and CGRP could provide the anatomical basis for the antipruritic efficacies of H₄R antagonists.

Detection of the H₄R on the soma of DRG neurons and the spinal cord further supports the interesting possibility that these receptors may have a role in the mediation of nociception. There is numerous data to support this potential role; the selective H₄R antagonists VUF 6002 and JNJ 7777120 were shown to have high efficacy in the attenuation of acute inflammatory pain. In this model, the drug reduced paw oedema and hyperalgesia induced by the subplantar injection of carrageenan (Coruzzi et al., 2007). JNJ777120 was shown to be efficacious in various models of inflammatory pain including Complete Freund's adjuvant, osteoarthritis, carrageenan thermal hyperalgesia (Hsieh et al., 2010a) and non-inflammatory such

as the skin incision model. JNJ777120 effectively reduces hyperalgesia in the chronic constriction and spinal nerve ligation models of neuropathic pain in the rat (Hsieh et al., 2010a).

3.5.3 Functional expression of the rat H₄R in the DRG

The H₄R mediates its effects by coupling to G α i/o G-proteins. Activation of the H₄R results in calcium mobilisation from intracellular stores (Hofstra et al., 2003). Due to the antinociceptive and antipruritic effects of H₄R antagonists in the absence of mast cells, the functional presence of the H₄R on sensory neurons was hypothesised. In the present study, it has been demonstrated that the H₄R is functionally expressed on sensory neurones using the potent highly selective agonist VUF 8430. VUF 8430 is a full agonist at human and rodent H₄R in both endogenous and recombinant H₄R expressing systems (Lim et al., 2009). Our results show that VUF 8430 induced a [Ca²⁺] influx in primary rat DRG neurons, which were sensitive to the H₄R selective antagonist, JNJ 7777120. VUF 8430 increased the normalised calcium intensity in a dose-related manner, demonstrating functional expression of the rat H₄R in the DRG. VUF 8430 displayed a pEC₅₀ value of 7.3 at the rat H₄R. However, this differs slightly from the pEC₅₀ 6.9 obtained by Lim *et al.* (2009). The difference could be due to the systems used; our data were collected using primary rat DRG cultures while Lim *et al.* used SK-N-MC cells stably transfected with the rat H₄R. These findings further corroborate those of Rosbbach *et al.* (2011) and, Connelly *et al.* (2009) Strakhova *et al.* (2009) which demonstrated the functional expression of the H₄R in the mammalian CNS and the hypothesis that the H₄R is involved in the transmission of itch and pain. Whole cell voltage recordings taken from layer IV somatosensory cells in adult mouse brain slices demonstrated that the H₄R agonist 4-Methylhistamine induced a reversible hyperpolarisation in the majority of neurons tested (Connelly et al., 2009) further supporting a functional role of the central H₄R.

The H₄R appears to be present on both peptidergic C and A δ fibres innervating the blood vessels and dermis of the skin, DRG and spinal cord. In contrast, the closely related H₃R was absent on the substance P positive putative C fibres (Cannon et al., 2007a), and immunoreactivity was only observed on CGRP positive putative A δ fibres

in cutaneous innervation. The neurons were primarily medium to large DRG neurons. Consistent with spinal H₃R inhibition of hyperalgesia, H₃R immunoreactivity was observed on small calibre CGRP-positive fibres ramifying the superficial lamina of the dorsal horn. The H₃R is a postsynaptic inhibitory heteroreceptor and administration of H₃R agonists has been shown to exhibit anti-inflammatory and anti-nociceptive properties, through inhibition of neuropeptides release. This mechanism may be important in controlling the extent of inflammation that occurs during injury, and subsequently released CGRP and substance P. It is possible that activation of the H₃R could attenuate the inflammatory effects induced by CGRP and substance P release and H₄R activation.

Our results show the presence of the H₄R on peripheral unmyelinated C- and A δ - and central primary afferents. This suggests both peripheral and central acting sites for H₄R antagonists that have antinociceptive and antipruritic effects. Previous studies showed that 4-Methylhistamine induced [Ca²⁺] in a subset of capsaicin-sensitive DRG neurones; capsaicin sensitivity is an essential characteristic of a major population of nociceptive sensory neurons, most of which are C-fibres but a small population are A δ fibres (Rosbach et al., 2011). The observed increase in free intracellular [Ca²⁺] observed in the DRG is related to capsaicin sensitivity because histamine induced [Ca²⁺] influxes in sensory neurones from wild type but not TPRV^{-/-} mice. Similarly, pre-treatment with a TPRV antagonist resulted in significantly attenuated scratching behaviour in response to histamine application (Shim et al., 2007).

This chapter provided an anatomical and functional basis for H₄R in rodent sensory neurotransmission. The next chapter investigates changes in H₄R expression in a range of acute and chronic inflammatory and neuropathic pain models.

Chapter 4

DIFFERENTIAL EXPRESSION OF THE HISTAMINE H₄ RECEPTOR IN ACUTE INFLAMMATORY AND NEUROPATHIC PAIN CONDITIONS

4.1 Objectives

To quantify the changes in histamine H₄ receptor expression during acute inflammatory and neuropathic pain conditions. The aim of this chapter was to perform a series of western blotting experiments to help understand the heterogeneity of H₄ receptor expression during different pain states.

4.2 Introduction

The histamine H₄ receptor, which was cloned in 2000 (Oda et al. 2000) is the latest most recently identified member of the histamine receptor family of G protein-coupled receptors with potential functional implications in inflammatory diseases. The H₄ receptor is primarily expressed in cells of the haematopoietic system such as eosinophils, mast cells and dendritic and T cells and has been postulated to possess immunomodulatory functions in these cells such as activation, migration, and production of chemokines and cytokines (Hofstra et al., 2003). In human eosinophils, activation of the H₄ receptor can induce an increase in intracellular calcium levels as well as cytoskeletal shape changes (Barnard et al., 2008). These findings support the participation of the H₄ receptor in immunity and inflammation and there are many *in vivo* studies that support this. For example in models of neutrophilia (Takeshita et al., 2003), allergic conjunctivitis (Varga et al., 2005), allergic rhinitis (Takahashi et al., 2009), asthma and allergic dermatitis (Ohsawa and Hirasawa, 2012).

Histamine appears to play a complex part in pain modulation as its release from mast cells is an established mediator of acute allergic reactions and chronic inflammation (Thurmond et al., 2004a). The relationship between the H₄ receptor and circulating immune cells and cytokine production is thought to participate in both the mechanism of inflammation and maintenance of inflammation via the H₄ receptor. H₄ receptor expression has also been reported in peripheral nerves (Nakaya et al., 2004), the neurons of the submucous plexus (Breunig et al., 2007) and

the close association between mast cells, microvessels, and sensory fibres has been confirmed (Alving et al., 1991). It is therefore highly possible that histamine alongside other mast cell products such as prostaglandins may contribute to pain sensation via the H₄ receptor.

A recent study suggested that H₄ receptor might be involved in the early phase of acute carrageenan-induced inflammation in the rat (Coruzzi *et al.* 2007). This study showed that the highly selective H₄ receptor antagonist JNJ 7777120 greatly reduced oedema in the rat model of acute inflammatory pain. Detection of H₄ receptors in the spinal cord and on cell bodies of DRG neurons also raises an intriguing possibility that these receptors may potentially have a role in mediation of nociception (Strakhova et al., 2009). Immunohistochemical detection in Chapter 3 revealed that H₄ receptors are present on the soma of sensory neurons with more intense staining observed in the small and medium diameter cells and lumbar spinal cord, primarily within lamina I and II, where the pattern suggests localization with primary afferent neurons from the dorsal root ganglia (DRG) (Strakhova et al., 2009). A study by Hsieh *et al.* (2010) showed the attenuation of hyperalgesia in rat models of inflammatory and neuropathic pain by the selective H₄ receptor antagonist JNJ 7777120, suggesting a role for this receptor in pain transmission (Hsieh et al., 2010a).

This chapter will describe the methods used to create the preclinical inflammatory and neuropathic pain models. Quantitative immunoblotting will be used to quantify the histamine H₄ receptor in sham treated, neuropathic and in the inflammatory models. The antibody used to label the histamine H₄ receptor is the anti-human H₄ receptor antibody, which recognises the C-terminal tail of the receptor (374-390) amino acid sequence (Shenton, 2007).

4.3 Methods

4.3.1 *In vivo* models of inflammatory pain

4.3.1A Rat model of Complete Freund's Adjuvant-induced (CFA) acute inflammatory pain

This procedure was done by our collaborators at GSK and they provided us with the tissue samples. Adult male Wistar rats (180-200 g) were used. Inflammatory hyperalgesia was induced in by intraplantar injection of 150 µl of Freund's complete adjuvant (containing 1 mg/ml *Mycobacterium tuberculosis*), into the plantar surface of the right hind paw. Sham control animals were injected with a saline solution. 16 days post-injection the rats were sacrificed by cervical dislocation (n=4 CFA, n=4 Sham) and spinal cords dissected and either snap frozen on dry ice or fixed in 4% paraformaldehyde until processing.

4.3.1B Behavioural testing

On the day of testing, the Hargreaves test was used to assess the acute nociceptive thermal threshold and induction of inflammatory pain. Animals were placed in a Perspex box resting on an elevated glass plate maintained at 30°C. Following an acclimation period, a high intensity infra-radiation (IR) source was placed directly beneath the hind paw. A trial was started by pressing a key on the IR source. The average time over three trials for the rat to remove the paw from the thermal stimulus was recorded in seconds as the paw withdrawal latency (PWL), and the IR source switched off with the reaction time counter stopped. The intensity of the beam was set to produce basal PWLs of 10-12 s. A maximal PWL of 20 s was used to prevent excessive tissue damage due to repeated application of the thermal stimulus. The withdrawal latency to the nearest 0.1 s was automatically determined and recorded. This was repeated three times and the average values determined.

In the contralateral paw, paw withdrawal latency (comparable with historical data), of less than 5 s was considered an exclusion criterion in behavioural studies. All CFA animals in this study would have been utilised for pharmacological testing if a behavioural study were being carried out. The spinal cords and paw skin from the

plantar surface was dissected following cervical dislocation, 24 hours after CFA injection and snap frozen in isopentane (Sham n=2, CFA n=5).

4.3.2 *In vivo* models of neuropathic pain

4.3.2A Rat Chronic Constriction injury-Induced (CCI) model of neuropathic pain

This procedure was done by our collaborators at Pfizer and they provided us with the tissue samples.

Adult male Wister rats (210-235 g) were used. As previously described in the method of Bennet and Xie (Bennett and Xie, 1988); the right sciatic nerve was isolated at mid-thigh level and loosely ligated by 3 chromic gut (5-0) ties separated by an interval of 1 mm.

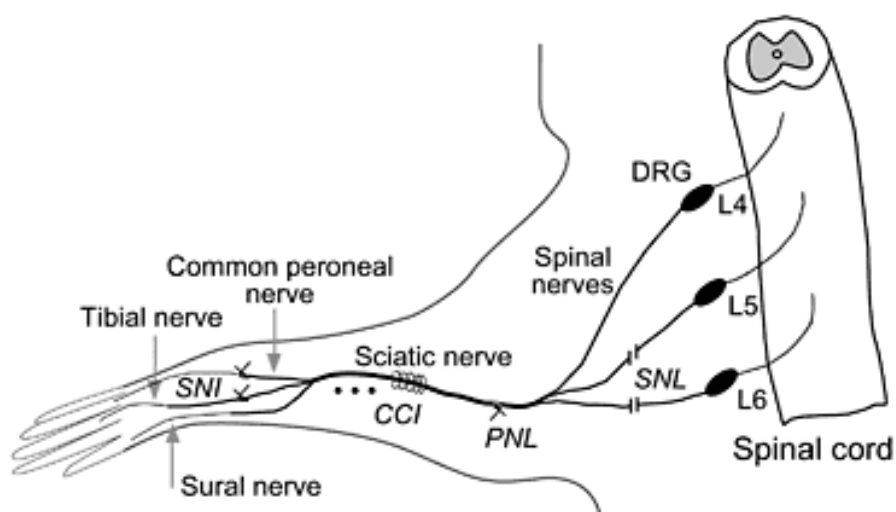


Figure 4.1 The diagram illustrates the location of the ligatures on the sciatic nerve. The sciatic nerve innervates the hind paw and its cell bodies of the sciatic nerve lie in dorsal root ganglia located in L4, L5 and L6. [Chronic constriction injury (CCI), partial nerve ligation (PNL), spared nerve injury (SNI), spinal nerve ligation (SNL)] (Ueda, 2006).

4.3.2B Behavioural testing and stimulus presentation

24 hours after surgery, static allodynia scores were taken using the method of Chaplan *et al.* (Chaplan *et al.*, 1994) using calibrated Von Frey filaments ranging from 0.4-15 g. On the day of testing, rats were placed in a plastic cage with a wire mesh floor that allowed access to the paws. Behavioural accommodation was allowed for approximately 15 minutes until cage exploration and grooming has ceased. The area tested was the mid-plantar right hind paw in the sciatic nerve distribution, avoiding the less sensitive foot-pads. The paw was touched with 1 of a series of 8 von Frey hairs with logarithmically incremental stiffness (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50 and 15.10 g). The von Frey hair was presented perpendicular to the plantar surface with sufficient force to induce a slight buckling against the paw, where it was held for approximately 6-8 s. The stimuli were presented at intervals of several seconds, allowing resolution of any behavioural responses to previous stimuli.

A positive response was noted if the paw was sharply withdrawn. Flinching immediately upon removal of a hair was also considered a response. Based on observations on normal, un-operated and healed sham-treated rats, the cut off of a 15.10 g von Frey hair was selected as the upper limit since stiffer hairs tended to raise the entire limb rather than to buckle, substantially changing the nature of the stimulus (Chaplan *et al.*, 1994).

4.3.2C Testing paradigm

Von Frey hairs were presented in ascending order of strength 24 hours after CCI. Each hair was presented 10 times and the number of positive responses multiplied by 10 was recorded as the percent response. For each rat, ascending stimuli were tested either until the maximum stimulus of 15.10 g was reached or until hair strength was reached that caused 100% response.

The 50% paw withdrawal threshold was determined using the Dixon method (1980). Testing was initiated with the 2 g hair and stimuli were presented in consecutive fashion whether ascending or descending. In the absence of paw withdrawal to the initially selected von Frey hair, a stronger stimulus was presented; in the event of

paw withdrawal, a weaker stimulus was chosen. According to Dixon, optimal threshold calculation requires 6 responses in the immediate vicinity of the 50% threshold. Since the threshold is unknown, strings of similar responses may be generated as the threshold is approached from either direction. Rats with a 50% paw withdrawal threshold (PWT) below 4g on the ipsilateral paw were considered alloydnic.

The paw skin, lumbar spinal cords and sciatic nerve from the CCI's were dissected following cervical dislocation, 24 hours after CCI induction and frozen to be used in western blots.

4.4 Results

Analysis of H₄ receptor expression in the rat model of acute inflammatory pain

4.4.1A Baseline PWLs of the control and CFA treatment groups

Initial experiments confirmed induction of inflammatory thermal hyperalgesia through the Hargreaves test.

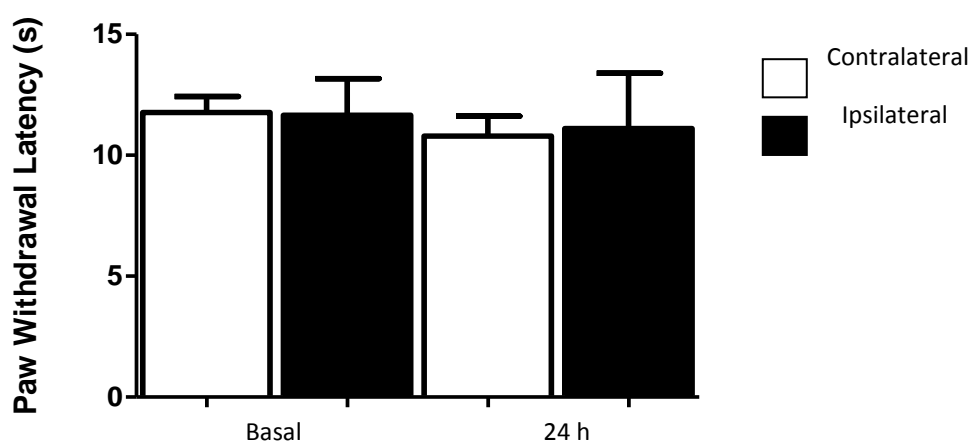


Figure 4.2 Effect of saline injection on paw withdrawal latency in the ipsilateral and contralateral hind paw of rats

The Hargreaves test was used to assess the nociceptive thermal threshold and induction of inflammatory pain. Animals were placed in a Perspex box and following an acclimation period, a high intensity infra-radiation (IR) source was placed directly beneath the hind paw. A trial was started by pressing a key on the IR source. When the animal felt pain and withdrew their hind paw, the IR source switched off and the reaction time counter stopped. The withdrawal latency to the nearest 0.1 second was automatically determined and recorded. This was repeated three times and the average values determined.

There was no difference in the paw withdrawal latencies to thermal stimulation obtained between the two groups.

4.4.1B Paw withdrawal latencies following saline and CFA treatment

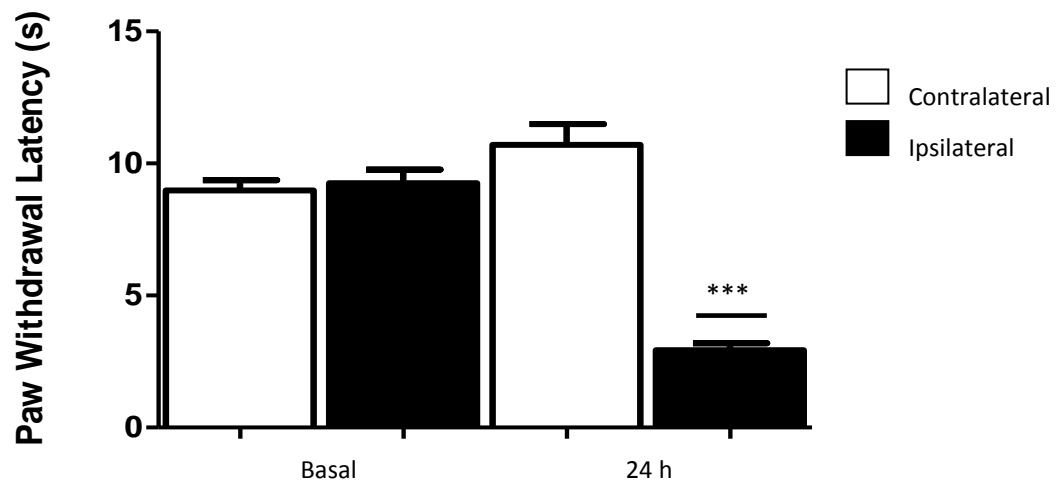


Figure 4.3 Effect of Freund's complete adjuvant on paw withdrawal latency in the ipsilateral and contralateral hind paw of rats.

Following CFA treatment, the paw withdrawal latency was markedly reduced in the ipsilateral hind paw after CFA injection, $p \text{ value} \leq 0.0001$. Statistical significance was determined through GraphPad Prism, using the Student's T-test, where $p \leq 0.05$ was considered to show significance. (CFA $n=10$, sham $n=4$)

4.4.2 Effect of CFA treatment on the expression of the H₄ receptor in the contralateral paw skin of CFA and saline treated rats

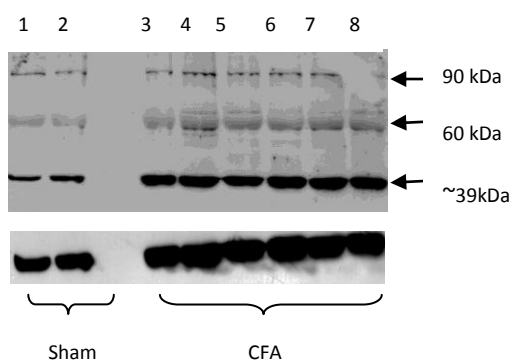


Figure 4.4 Anti-hH₄ immunoreactivity with protein extractions from contralateral paw skin of Complete Freund's adjuvant treated and control rats. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:2000)

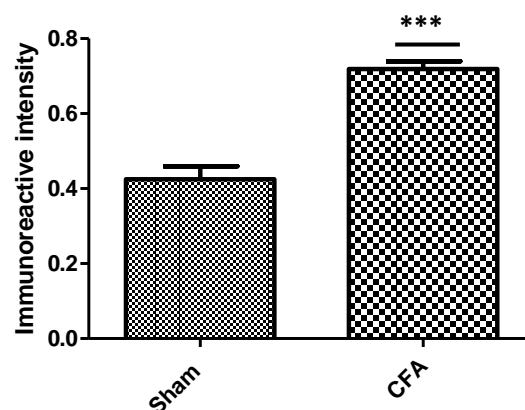


Figure 4.5 Mean reactive immunointensity of the Mr 39,000 rH₄R bands detected, using the anti-hH₄R antibody in the contralateral paw skin of sham and CFA treated rats. P value=0.0004.

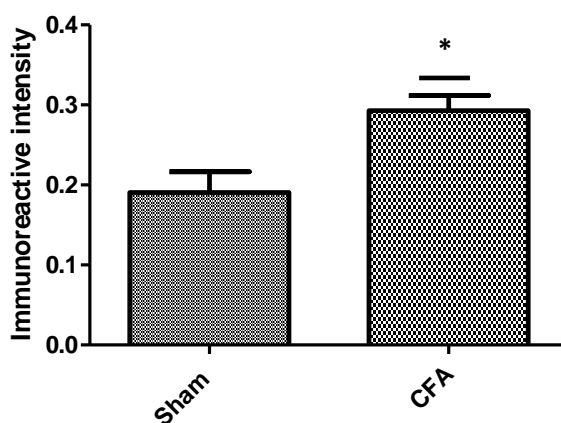


Figure 4.6 Mean reactive immunointensity of the Mr 60,000 rH₄R bands detected, using the anti-hH₄R antibody in the contralateral paw skin of sham and CFA treated rats. P value=0.0322.

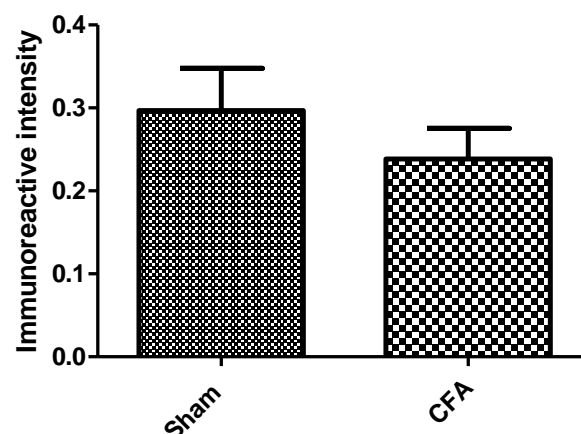


Figure 4.7 Mean reactive immunointensity of the Mr 90,000 rH₄R bands detected, using the anti-hH₄R antibody in the contralateral paw skin of sham and CFA treated rats. P value=0.4474.

Approximately 50 µg of protein/ well was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 µg/ml concentration). Mean ± SD [sham n=2, CFA=6]. Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β-actin. Data show the mean immunoreactive intensity ±SD for n=3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test, where p≤0.05 was considered to show significance.

Figure 4.4 shows the three immunoreactive bands detected in rat paw skin using the anti-hH₄ antibody. The bands represent the full length H₄ receptor monomer migrating at approximately Mr 39, 000 and a dimeric form of the receptor migrating at Mr 60, 000. The largest fragment migrating at approximately at Mr 90,000 is likely to represent the oligomers of the receptor.

Figure 4.5 shows there were significant differences in the immunoreactive bands detected at approximately Mr 39,000 with the anti-hH₄ receptor antibody, between the CFA and saline treated rats. The bands detected in the CFA treated rats had significantly higher immunoreactivity, suggesting that during CFA induced peripheral inflammatory pain; there is a bilateral increase in the expression of the full length H₄ receptor in the skin of rats.

Figure 4.6 shows a significant increase in the immunoreactive bands detected at approximately Mr 60,000 with the anti-hH₄ receptor antibody, between the CFA and saline treated rats.

Figure 4.7 There was no significant difference in the expression of the Mr 90 000 rH₄ receptor in the contralateral paw skin of saline and CFA animals.

4.4.3 Effect of Complete Freund's adjuvant treatment on the expression of the H₄ receptor ipsilateral (treated) paw skin of adjuvant and saline treated rats

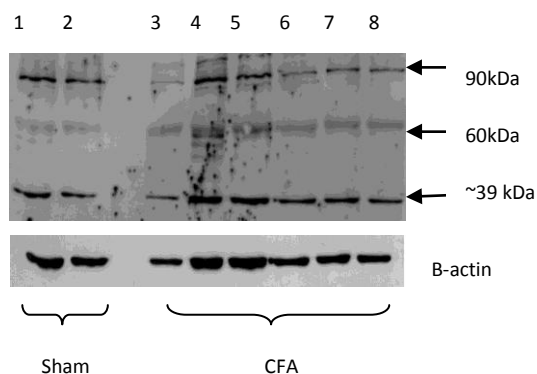


Figure 4.8 Anti-hH₄ immunoreactivity with protein extractions from ipsilateral paw skin of Complete Freund's adjuvant treated and control rats. Lower panel shows the corresponding β -actin, probed with monoclonal mouse anti β -actin antibody (1:2000).

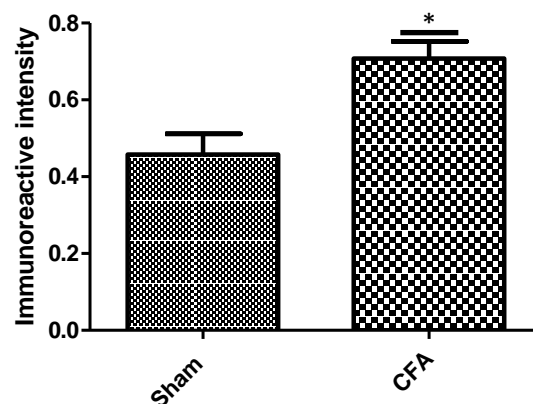


Figure 4.9 Mean reactive immunointensity of the Mr 39,000 rH₄R bands detected, using the anti-hH₄R antibody in the ipsilateral paw skin of sham and CFA treated rats. P value=0.0255.

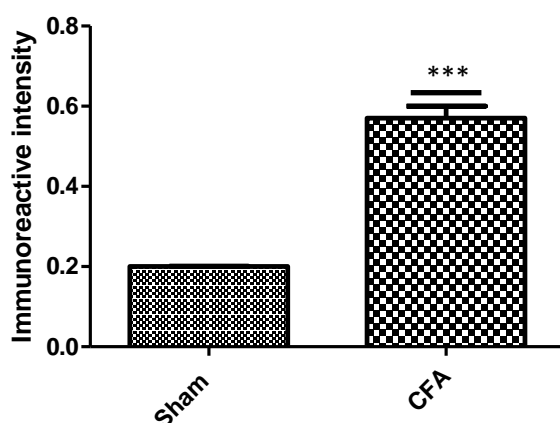


Figure 4.10 Mean reactive immunointensity of the Mr 60,000 rH₄R bands detected, using the anti-hH₄R antibody in the ipsilateral paw skin of sham and CFA treated rats. P value=0.0005.

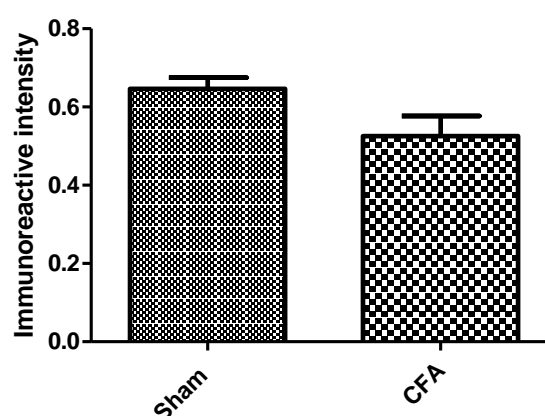


Figure 4.11 Mean reactive immunointensity of the Mr 90,000 rH₄R bands detected, using the anti-hH₄R antibody in the ipsilateral paw skin of sham and CFA treated rats. P value=0.2522.

Approximately 50 μ g of protein/ well was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 μ g/ml concentration). Mean \pm SD [sham n=2, CFA=6]. Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity \pm SD for n = 3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test, where $p \leq 0.05$ was considered to show significance

Figure 4.8 shows three immunoreactive bands detected in rat skin using the anti-hH₄ antibody represent the H₄ receptor monomer migrating at approximately Mr 39, 000 and a dimeric form of the receptor migrating at Mr 60, 000. The largest fragment migrating at approximately at Mr 90,000 is likely to represent the oligomers of the receptor

Figure 4.9 shows there was a significant difference in the immunoreactive bands detected at approximately Mr 39,000 with the anti-hH₄ receptor antibody, between the CFA and saline treated rats. The bands detected in the CFA treated rats had significantly higher immunoreactivity suggesting that during CFA induced peripheral inflammatory pain; there is an increase in the expression of the H₄ receptor monomer in the skin of rats.

Figure 4.10 shows there were significant differences in the immunoreactive bands detected at approximately Mr 60,000 with the anti-hH₄ receptor antibody, between the CFA and saline treated rats.

Figure 4.11 shows there was no significant difference in the expression of the Mr 90 000 rH₄ receptor in the contralateral paw skin of saline and CFA treated animals.

4.4.4 Immunoreactivity of the anti-hH₄ (374-390) on spinal cord tissue obtained from acute Freund's Complete Adjuvant and saline treated rats

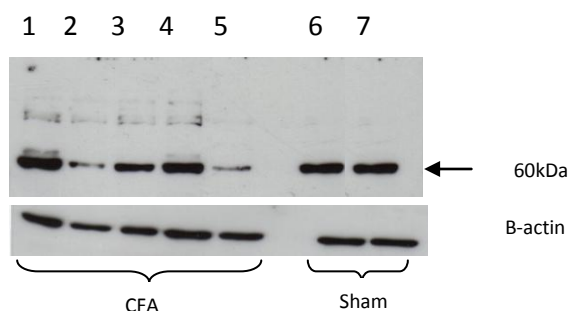


Figure 4.12 Immunoblot of spinal cord sections obtained from the lumbar section of Freund's complete adjuvant treated and saline treated rats. Immunoreactive bands were detected with the anti-hH₄ receptor antibody and normalised to β -actin. Lanes **1- 5** represent CFA treated animals and lanes **6-7** represent saline treated animals

Approximately 50 μ g of protein/ well from whole spinal was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 μ g/ml concentration). The antibody detected one band of immunoreactivity at Mr 60,000. Lower panel shows the corresponding β -actin, probed with monoclonal mouse anti β -actin antibody (1:2000).

Figure 4.12 shows the immunoreactivity detected when the anti-hH₄ (374-390) antibody was used to probe the rat lumbar spinal cord homogenates. The band detected at Mr 60,000 is consistent with the molecular weight corresponding to the size of the H₄ receptor dimer. On the other hand, the molecular weight corresponding to the monomer was not detected.

4.4.5 Mean reactive immunointensity of the Mr 60, 000 rH₄R bands detected using the anti-hH₄R antibody, in spinal cord tissue obtained from acute Freund's Complete Adjuvant and saline treated rats

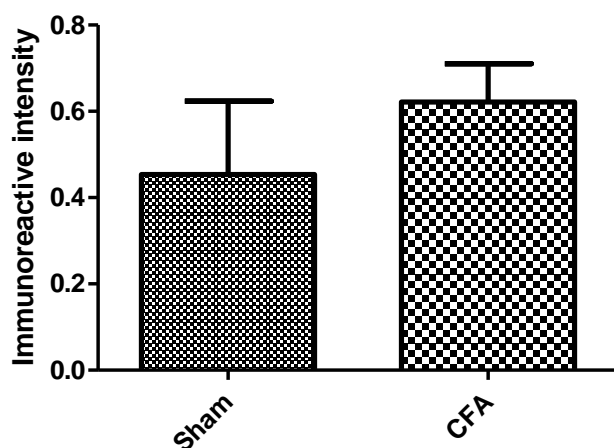


Figure 4.13 Comparing expression of the Mr 60, 000 rH₄R in the lumbar spinal cord of CFA treated and saline treated rats probed with anti-hH₄ antibody. P value=0.3518. Mean \pm SD [sham n=2, CFA=6]. Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity \pm SD for n = 3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test from the generated p value, where $p \leq 0.05$ was considered to show significance.

Figure 4.13 shows that there was no significant difference in the immunoreactive banding detected between the complete Freund's Adjuvant-treated rats and the saline treated sham controls. This suggests that there is no significant difference in H₄R expression in the spinal cord during the acute phase of inflammatory pain.

Analysis of H₄ expression in the rat model of acute neuropathic pain

4.4.6 The effect of chronic constriction injury of the sciatic nerve on paw withdrawal latency in the ipsilateral and contralateral hind paw of rats

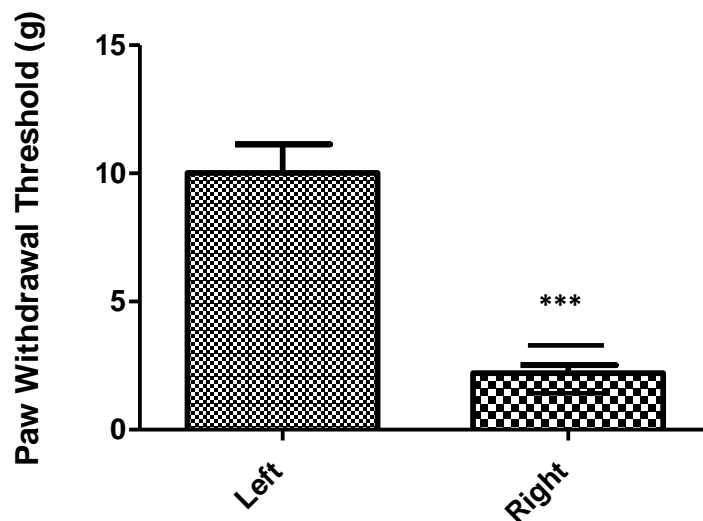


Figure 4.14 Effect of chronic constriction of the sciatic nerve on paw withdrawal threshold in the ipsilateral (right) and contralateral (left) hind paws of rats.

Figure 4.14 shows that following CCI, the paw withdrawal threshold in the ligated hind limb were significantly reduced, compared to the sham treated hind limb. (CCI n=8, sham n=4)

Statistical significance was determined through GraphPad Prism, using the Student's T-test, where $p \leq 0.05$ was considered to show significance.

4.4.7 Immunoreactivity of the anti-hH₄ (374-390) on paw skin tissue obtained from ipsilateral plantar paw of CCI –induced neuropathic pain model (CCI) and Sham rats

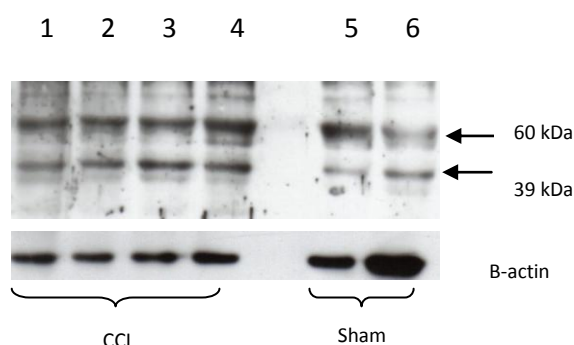


Figure 4.15 Immunoreactivity of anti-hH₄R on skin from the ipsilateral paw skin of CCI-induced neuropathic pain and control rats

Lanes **1-4** represent CCI animals, lanes **5-6** sham animals. Proteins extracted from the ipsilateral and ipsilateral hind paw skin were subjected to immunoblotting and immunoblots probed with the anti-hH₄ (374-390) antibody at 1 µg/ml.

Approximately 50 µg of protein/ well was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 µg/ml concentration). The antibody detects two major species in rat skin, M_r 39,000 and 60,000. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:2000).

In figure 4.15, the two immunoreactive bands detected in rat paw skin using the anti-hH₄ antibody represent the rH₄R monomer migrating at approximately M_r 39 000 and a dimeric version of the receptor migrating at M_r 60,000.

4.4.8 Mean reactive immunointensity of rH₄R bands detected using the anti-hH₄ receptor antibody, in the ipsilateral paw skin of CCI-induced neuropathic pain compared to the sham treated rats

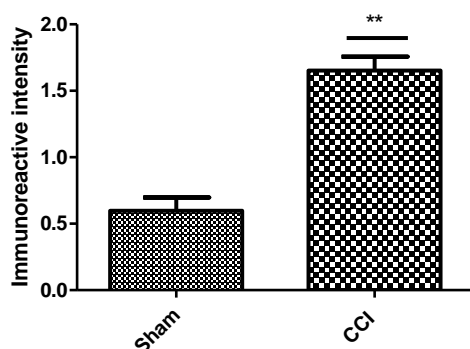


Figure 4.16 Mean reactive immunointensity of the Mr 39, 000 rH₄R bands detected in the ipsilateral paw skin of CCI rats compared to the saline treated sham controls. P value=0.0033.

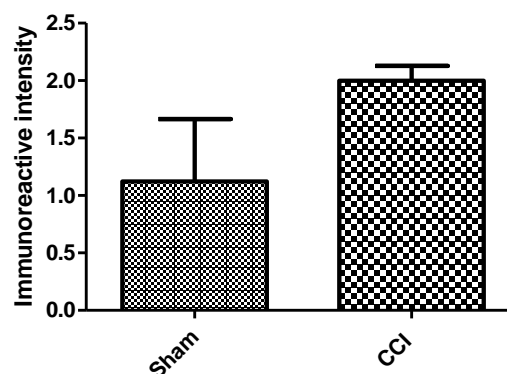


Figure 4.17 Mean reactive immunointensity of the Mr 60, 000 rH₄R bands detected in the ipsilateral paw skin of CCI rats compared to the saline treated sham controls. P value=0.0849

Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity. Statistical significance was determined through GraphPad Prism, using the Student's T-test from the generated p value, where $p \leq 0.05$ was considered to show significance. Mean \pm SD [Sham n=2, CCI=4] for n = 3 determinations

Figure 4.16 shows significant differences in the immunoreactive banding of the Mr 39, 000 rH₄ receptor detected, in the CCI rats compared to the saline treated sham controls. The bands detected in the CCI treated rats had significantly higher immunoreactivity, suggesting that during CCI induced peripheral neuropathic pain; there is an increase in the expression of the H₄ receptor monomer in the skin from the plantar surface of the hind paw of rats.

Figure 4.17 shows that there were no significant differences in the expression of the Mr 60,000 H₄ receptor dimer in the skin from the plantar surface of the ipsilateral paw.

4.4.9 Immunoreactivity of the anti-hH₄ (374-390) on paw skin tissue obtained from contralateral paw of CCI-induced neuropathic pain model and Sham rats

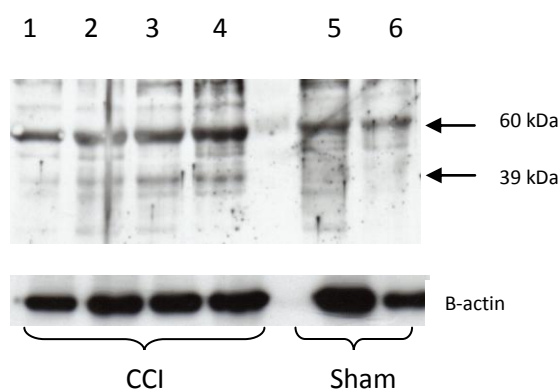


Figure 4.18 Immunoreactivity with protein extractions from contralateral paw skin of CCI-induced neuropathic pain and control rats

Lanes **1-4** represent CCI animals, lanes **5-6** sham animals. Proteins extracted from the contralateral and ipsilateral hind paw skin were subjected to immunoblotting and immunoblots probed with the anti-hH₄ (374-390) receptor at 1 µg/ml.

Approximately 50 µg of protein/ well was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 µg/ml concentration). The antibody detects two species in rat skin, M_r 48,000 and 60,000. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:2000).

Figure 4.18 shows the immunoreactive bands detected represent the H₄R monomer migrating at approximately Mr 39, 000 and the dimer migrating at Mr 60,000.

4.4.10 Mean reactive immunointensity of the rH₄R bands detected using the anti-hH₄ receptor antibody, in the contralateral paw skin of CCI-induced neuropathic pain rats

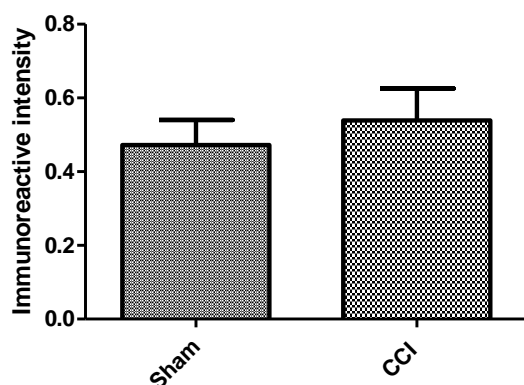


Figure 4.19 Mean reactive immunointensity of the Mr 39, 000 rH₄R bands detected in the ipsilateral paw skin of CCI rats compared to the saline treated sham controls. P value=0.6465.

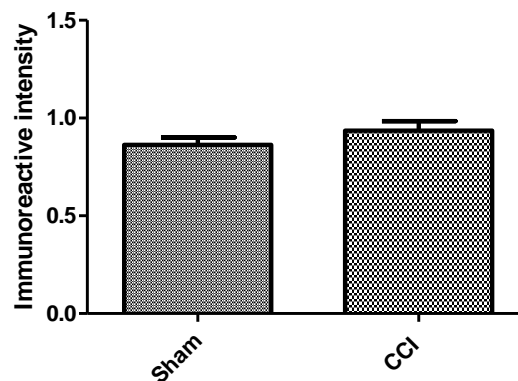


Figure 4.20 Mean reactive immunointensity of the Mr 60, 000 rH₄R bands detected in the ipsilateral paw skin of CCI rats compared to the saline treated sham controls. P value=0.3896.

Comparing expression of the Mr 39, 000 and Mr 60, 000 rH₄R in the plantar paw skin of CCI and saline treated rats, probed with anti-hH₄ antibody. Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity \pm SD for n = 3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test from the generated p value, where $p \leq 0.05$ was considered to show significance. [Sham n=2, CCI=4].

Figure 4.19 shows that there were no significant differences in the immunoreactive banding of the Mr 39,000 rH₄ receptor detected in the CCI rats compared to the saline treated sham controls.

Figure 4.20 shows that there were no significant differences in the immunoreactive banding of the Mr 60 000 rH₄ receptor detected in the CCI rats compared to the saline treated sham controls.

4.4.11 Immunoreactivity of the anti-hH₄ (374-390) on lumbar spinal cords obtained from CCI –induced neuropathic pain (CCI) rats

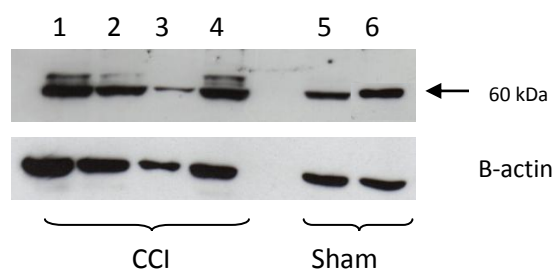


Figure 4.21 Anti-hH₄ immunoreactivity with protein extractions from contralateral paw skin of CCI-induced neuropathic pain and control rats

Lanes **1-4** represent CCI animals, lanes **5-6** sham animals. Proteins extracted from the contralateral and ipsilateral hind paw skin were subjected to immunoblotting and immunoblots probed with the anti-hH₄ (374-390) receptor at 1µg/ml.

Approximately 50 µg of protein/ well were loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 µg/ml concentration). The antibody detects one major species in rat spinal cord at Mr 60,000. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:2000).

Figure 4.21, the anti-hH₄ (374-390) was used to probe the rat lumbar spinal cord homogenates. The banding detected at Mr 60,000 is consistent with the rH₄ receptor dimer. The molecular weight corresponding to the monomer was not detected.

4.4.12 Mean reactive immunointensity of the Mr 60, 000 rH₄R bands detected in the lumbar spinal cord of CCI-induced neuropathic pain model

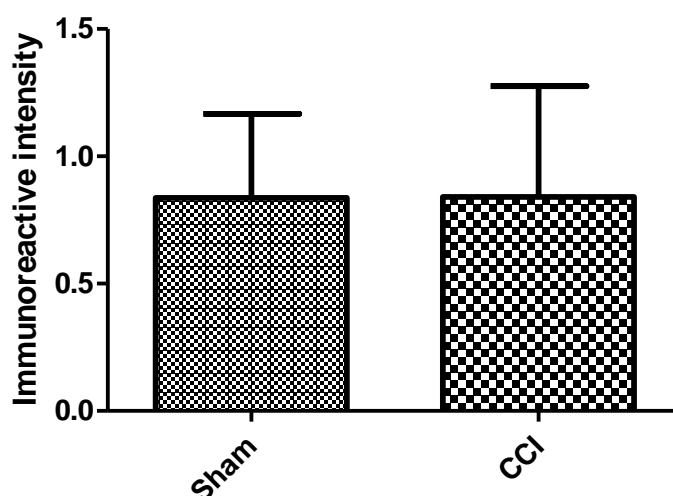


Figure 4.22 Comparing expression of the Mr 60, 000 rH₄R in the lumbar spinal cord of CCI and saline treated rats, probed with anti-hH₄ antibody. P value=0.9798. Mean \pm SD [Sham n=2, CFA=4]. Immunoreactive bands detected with the anti-H₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity \pm SD for n = 3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test from the generated p value, where $p \leq 0.05$ was considered to show significance.

Figure 4.22 shows that there were no significant differences in the immunoreactive banding of the Mr 60,000 rH₄ receptor detected in the CCI rats compared to the saline treated sham controls.

4.4.13 Immunoreactivity of the anti-hH₄ (374-390) receptor antibody on the sciatic nerve obtained from CCI –induced neuropathic pain (CCI) rats

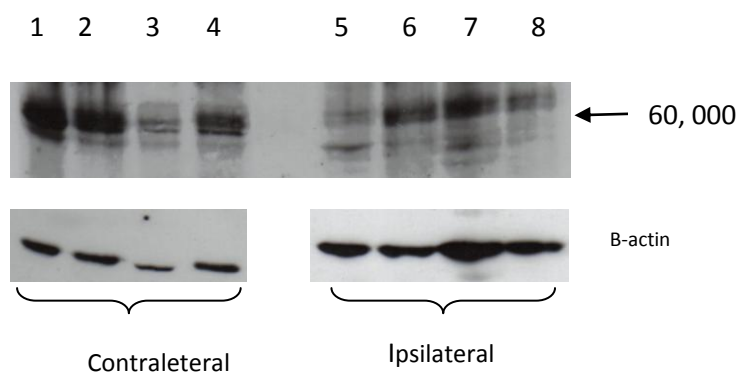


Figure 4.23 Anti-hH₄ immunoreactivity with protein extractions from the sciatic nerve of the CCI rats

Lanes **1-4** represent sciatic nerve samples from the contralateral limb and lanes **5-8** represent the sciatic nerve from the ipsilateral limb of CCI-induced neuropathic pain models. Proteins extracted from the contralateral and ipsilateral hind paw skin were subjected to immunoblotting and immunoblots probed with the anti-hH₄ (374-390) receptor at 1µg/ml.

Approximately 50 µg of protein/ well was loaded onto a 10% separating gel. Once transferred the membrane was probed with affinity purified anti-hH₄ antibody (1 µg/ml concentration). The antibody detects one major band in the rat sciatic nerve at Mr 60,000. Lower panel show the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:2000).

Figure 4.23, the anti-hH₄ (374-390) was used to probe the rat sciatic nerve homogenates. The banding detected at Mr 60,000 is consistent with the rH₄ receptor dimer, while the monomer was not detected.

4.4.14 Mean reactive immunointensity of the Mr 60, 000 rH₄R bands detected in the sciatic nerve of CCI-induced neuropathic pain model

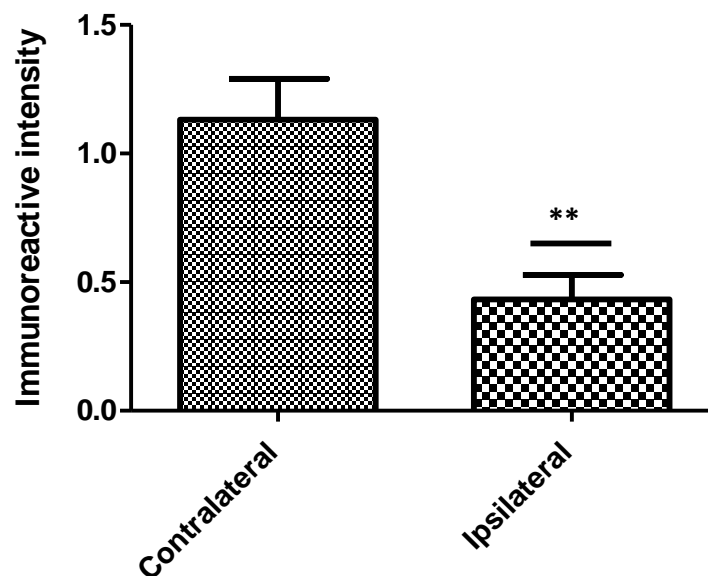


Figure 4.24 Comparing expression of the Mr 60, 000 rH₄R in the sciatic nerve of CCI rats, probed with anti-hH₄ antibody. P value=0.0019. Mean \pm SD [contralateral n=4, ipsilateral n=4]. Immunoreactive bands detected with the anti-hH₄R antibodies were normalized to β -actin. Data show the mean immunoreactive intensity \pm SD for n = 3 determinations. Statistical significance was determined through GraphPad Prism, using the Student's T-test from the generated p value, where $p \leq 0.05$ was considered to show significance

Figure 4.24 shows that there were significant differences in the immunoreactivity of between the sciatic nerve obtained from the contralateral and ipsilateral hind limb of the CCI rats. The banding detected at Mr 60,000 which is consistent with the rH₄ dimer was significantly reduced in the ipsilateral sciatic nerve.

4.4.15 Summary of results obtained through quantitative immunoblotting of tissue from acute inflammatory and neuropathic pain models

Rat model		Acute inflammatory pain	Acute neuropathic pain
Skin	Ipsilateral	↑	↑
	Contralateral	↑	-
Sciatic nerve	Ipsilateral	-	↓
	Contralateral	-	-
Spinal cord		No change	No change

Table 4.1 Summary the results obtained regarding H₄R expression in the skin and spinal cord of the acute inflammatory and neuropathic pain models

4.5 Discussion

The histamine H₄ receptor was initially reported in bone marrow, spleen, lung and small intestine but is largely found on cells of the haematopoietic system such as dendritic cells (Damaj et al., 2007, Gutzmer et al., 2005), eosinophils (Barnard et al., 2008) and mast cells (Oda, 2001) where it mediates chemotaxis (O'Reilly et al., 2002). Chapter 3 has shown that the H₄R is present in the central nervous system, leading to the hypothesis of potential role for the H₄ receptor in the transmission and maintenance of nociception and pain, respectively. H₄R expression was analysed in the skin from the plantar surface of the hind paw, and lumbar spinal cord. Herein we will evaluate H₄R expression during acute inflammatory and neuropathic pain. Inflammatory pain is the result of inflammatory mediators such as chemokines and leukocytes, whereas neuropathic pain is produced by injury to the central nervous system including peripheral nerves, dorsal root ganglion, spinal cord or brain (Milligan et al., 2003).

Acute inflammatory pain

Peripheral administration of Complete Freund's adjuvant (CFA) creates an inflammatory pain model characterised by spontaneous pain, heat hyperalgesia and mechanical allodynia (Raghavendra et al., 2004). Consistent with previous data (Chen et al., 2012), injection of CFA into the plantar surface of the hind paw, caused a significant reduction in the paw withdrawal latency (in seconds) to mechanical stimuli during the Hargreaves test. The reduction in paw withdrawal observed in our model following CFA administration was unilateral, and is consistent with Complete Freund's adjuvant-induced acute inflammatory pain (Hsieh *et al.* 2010).

Quantification of the protein extracted from skin excised from the plantar hind surface during the acute phase of inflammation revealed an apparent, non-selective increase in H₄R expression in acute CFA inflammatory model of pain in both ipsilateral and contralateral skin but no change at spinal cord level. CFA induces a localised inflammatory reaction and recruitment of neutrophils, activated mast cells, Langerhan's cells and eosinophils. These cells express the H₄R which plays a role in migration and chemotaxis (Hofstra et al., 2003, Ling et al., 2004). The up-regulation

in H₄R expression observed in the protein extractions from the skin is possibly due to increased recruitment immune cells to the site of inflammation. Dendritic cells express the H₄R and are recruited to the inflamed site by chemokines in the microenvironment. Nerve terminals influence neurogenic inflammation through the generation of impulses, which release vasoactive neuropeptides substance P, CGRP and histamine at peripheral branches. H₄ receptor antagonism was shown to inhibit dendritic cell maturation through reduced expression of maturation markers, CD80, CD86 and CD40 in treated cells (Lundberga et al., 2011), supporting H₄R involvement. In addition, has been observed that H₄R levels in monocytes increase with the progression of inflammatory stimuli (Dijkstra et al., 2007).

The changes observed in the contralateral side were unexpected and could be a result of a systemic inflammatory response. On the other hand, the response could be neurogenic since substance P, CGRP and their receptors have been observed within the spinal cord and DRG contralaterally following CFA induced lesions. The contralateral responses seem to require the inflammatory stimulus to be of a certain magnitude before appearing. For example, adjuvant arthritis in the rat induced with intradermal injections of small doses of CFA around the tibiotarsal joint causes contralateral arthritis if the dose is increased from 75 to 150 µg (Shenker et al., 2003). These contralateral changes appear to be topologically precise. Decaris *et al* (1999) placed latex spears in the knee joint before examining the contralateral knee and both ankles for cartilage metabolism and histological damage and observed that only the contralateral knee showed a decrease in cartilage anabolism and signs of histological damage, and these were absent in both ankles (Decaris et al., 1999). This implies a neurogenic mechanism rather than systemic effect.

The contralateral changes are also stimulus-specific (if proinflammatory response is induced unilaterally, the contralateral response is proinflammatory) (Shenker et al., 2003) and are only a fraction of the original lesion. One hypothesized role is that contralateral mirror imaging phenomena can up-regulate protective proinflammatory responses in the contralateral limb in preparation for an insult that has already occurred (Herdegen et al., 1991). Biologically, this could be protective mechanism as the inflammatory response could be up-regulated and, should a

similar noxious insult appear on the contralateral side, then it could be dealt with more effectively.

The anti-hH₄R₍₃₇₄₋₃₉₀₎ was also used to detect changes in H₄R expression at the level of the spinal cord after acute inflammation. Western blot analysis of whole lumbar spinal cord from the acute inflammatory pain model shows no difference in expression of spinal H₄R in the CFA-treated acute model animals compared to the sham treated controls. This shows that inflammatory pain experienced during the acute phase may not be mediated by differential expression of the H₄R.

Acute neuropathic pain

Chronic constriction injury was produced by tying constrictive ligatures around the sciatic nerve, evoking intraneural oedema. In this model, hyperalgesia was present in the whole area of the damaged sciatic territory and neuropathic animals exhibit signs of increased mechanical sensitivity at the 4 g threshold. In the rats, chronic construction injury of the sciatic nerve produced a decreased in paw withdrawal latency to mechanical stimulation 24 hours after injury. The hyperalgesia is consistent with the development of mechanical allodynia and withdrawal thresholds can remain reduced for up to 38 weeks. (Lindenlaub and Sommer, 2000).

The results show that following CCI of the sciatic nerve, there was a selective increase in Mr 39 000 rH₄ receptor expression in skin from the ipsilateral the hind paw. The observed increase in H₄R expression in the CCI rats is possibly situated on peripheral afferent fibres, as expression was detected in Chapter 3. CCI induces a neurogenic inflammatory response, mediated by the release of neuropeptides from the nerve endings of antidromically acting nociceptive C fibres. Antidromic activation of unmyelinated primary afferents C-fibres produces the physiological characteristics of inflammation including plasma extravasation, vasodilatation and sensitisation of nociceptors (Levine et al., 1990).

Peripheral afferents are sensitised by the inflammatory mediators released from leukocytes which accumulate around the lesion of the injured peripheral nerve,

thereby contributing to the generation of neuropathic pain (Perkins and Tracey, 2000). Neutrophils transmigrate through the perineurium 2 mm proximally and distally to the injury (Perkins and Tracey, 2000). Several mediators attract and activate neutrophils at the site of injury including nerve growth factor, which is involved in both inflammatory and peripheral neuropathic hyperalgesia and leukotriene B₄ (Jankowski and Koerber, 2010). Neutrophils release prostaglandins which sensitize primary nociceptive afferents and contribute to nerve injury associated hyperalgesia (Syriatowicz et al., 1999).

The anti-hH₄ antibody detected the expression of the Mr 60,000 rH₄ receptor dimer in the sciatic nerve, which innervates nearly the whole skin of the lower limbs, muscles, biceps femoris, feet and legs. Quantitative analysis detected a selective reduction in the expression of the Mr 60,000 H₄ receptor in the ipsilateral sciatic nerve compared to the contralateral. Previous data from this model (Bennett and Xie, 1988) showed extensive demyelination and reduction in the diameter of the sciatic nerve 24 hours following surgery. The nerve damage produced in this model is due to chronic compression, a consequence of intraneural oedema, which is subsequent to partial occlusion of the nerve's vasculature. The force of the oedema opposes the ligature beneath, causing the nerve to self-strangulate (Bennett and Xie, 1988). Mast cells that reside in the nerve degranulate at the site of injury, releasing algescic mediators such as histamine, prostaglandins and pro-inflammatory cytokines like TNF α (Wagner and Myers, 1996) and IL-1 (Sommer et al., 1999) which contribute to neuropathic pain behaviour.

Following CCI-induced neuropathic pain there is no change in expression of the H₄ receptor in the whole spinal cord between the two groups. However, nerve injury causes changes in spinal cord neuropeptide expression. The selective loss of non-peptidergic IB₄ positive nociceptive afferents in the dorsal horn has been observed following peripheral nerve injury (Kim and Moalem-Taylor, 2011, Bailey and Ribeiro-da-Silva, 2006) and denotes substantial damage to non-peptidergic C-fibre nociceptor terminals entering the spinal cord. CGRP expression remains unchanged in the dorsal horn up to 70 days following sciatic nerve ligation (Bailey and Ribeiro-

da-Silva, 2006). Substance P produces thermal hyperalgesia and expression is initially enhanced in the DRG following sciatic nerve injury (Xu et al., 1996).

We have shown that the H₄R is differentially expressed in these models of acute inflammatory and neuropathic pain. There is an apparent non-selective increase in expression in acute CFA inflammatory model of pain in both ipsilateral and contralateral skin. This could be a result of the systemic effect of inflammatory cells. However, no changes in H₄R expression are observed in the spinal cord. Changes in receptor expression or function can occur in the chronic phase of inflammatory pain. Data obtained from our laboratory (Chaffey *et al.* unpublished) demonstrated that the presence of chronic pain in the CFA-induced inflammatory pain model was as due, in part, to up-regulated phosphorylation of the NMDA NR2B subunit. This was observed in the dorsal horn of the lumbar cord (Chaffey *et al.*), showing enhanced receptor activity at the site of afferent neuron entry into the spinal cord, where nociceptive afferents from the injured paw synapse with dorsal horn neurons. On the other hand, in the CCI model, there is a selective increase in H₄R expression at ipsilateral skin level but not in the contralateral. A reduction in H₄R expression in sciatic nerve ipsilateral versus contralateral is also observed, but no change at the level of spinal cord. This provides evidence for the potential functional role of histamine acting via the H₄R in pain. The peripheral and central localisation of the H₄ receptor makes it a possible therapeutic target for the amelioration of inflammatory pain processes.

These results further the understanding of H₄R expression in acute inflammatory and neuropathic pain conditions. The selective H₄ receptor antagonist JNJ 7777120 was shown to readily permeate through the BBB and exert analgesic effects on several animal models of inflammatory and neuropathic pain (Hsieh et al., 2010a). Examples of the *in vivo* inflammatory pain models used include the carrageenan-induced acute inflammation, complete Freund's adjuvant-induced chronic inflammation, zymosan-induced peritonitis (Takeshita et al., 2003) and the knee-joint osteoarthritic model. Similarly, Hsieh *et al.* (2010) provided the first evidence showing the effectiveness of JNJ 7777120 as an analgesic in the reduction of CCI- induced neuropathic pain in animal models. The anti-nociceptive effects of the H₄R antagonists could be

secondary to the anti-inflammatory actions of the antagonists. It is possible that the acute phase of inflammatory pain is mediated by influx of inflammatory leukocytes which release mediators of hyperalgesia.

In Chapter 5, we investigate the effects of H₄ receptor antagonism using the antagonist compound JNJ 7777120, on a murine model of experimental autoimmune encephalomyelitis. Current literature shows that the histamine H₄ receptor has a primary role in inflammatory functions, making it an attractive therapeutic target and the discovery of JNJ 7777120 as a selective and potent antagonist made it possible to elucidate some of the roles of the H₄ receptor in inflammatory processes (Thurmond et al., 2008a).

Chapter 5

The role of histamine H₄ receptor in Experimental Autoimmune encephalomyelitis (EAE)

5.1 Objectives

To elucidate the effects of histamine H₄ receptor antagonism with JNJ 7777120 on several parameters of the murine model of MOG₃₅₋₅₅ induced experimental autoimmune encephalomyelitis.

5.1.1 Introduction

In Chapter 4, we have shown the changes in H₄R expression in different inflammatory and neuropathic pain states, which indicated that antagonism of the receptor, may have beneficial therapeutic effects. Multiple Sclerosis (MS) is a chronic multicentric inflammatory, demyelinating disease of the CNS. Clinically, MS manifests with fatigue and disturbed function in the sensory, motor, bladder and bowel, cerebella, brainstem, optic nerves and cognitive realms. Morphologically, it is characterised by inflammation, demyelination, axonal loss and gliosis. The inflammatory lesions are characterized by high infiltration of various populations of cellular and soluble mediators of the immune system, such as T-and B cells and microglia (Moore and Esiri, 2011). Several models of CNS demyelinating disease exist but the most commonly used model is the Experimental Allergic Encephalomyelitis (EAE). This is induced by subcutaneous immunisation with myelin antigens emulsified in adjuvant, or adoptive transfer of encephalitogenic T cells into susceptible animals mainly mice (Stromnes and Goverman, 2006). In the majority of EAE susceptible rodent strains, disease presents clinically as ascending flaccid paralysis beginning with a limp tail and progressing to the hind and forelimbs (Stromnes and Goverman, 2006). This is referred to as “classic” EAE. The clinical signs observed in classic EAE reflect inflammation localised predominantly in the spinal cord (Robinson et al., 2010). **Central neuropathic pain** is a frequent symptom accompanying MS and pain syndromes include musculoskeletal pain, painful spasms, trigeminal neuralgia and central pain caused by sclerotic plaques (O'Connor et al.,

2008, Truini et al., 2012). However, pain is not present in all MS patients and the reasons for this are unknown. One theory suggests that a lesion in the CNS may result into a state of hyperexcitability and spinal cord injury (SCI) has been shown to increase spontaneous neuronal activity at the site of injury (Zeilig et al., 2012). Some studies in SCI indicate that lesion of the spinothalamic tract is necessary for the development of central pain whereas others have failed to find any difference in spinothalamic function between pain-free and patients with central pain. A study by Svendsen *et al.* reported that MS patients with pain frequently presented with cold allodynia and lower threshold for mechanical pressure than pain-free patients (Svendsen et al., 2005). These findings reflect a degree of hyperexcitability possibly caused by lesions of descending inhibiting pathways of the CNS or decreased sensory input to second or third order neurons (Davies et al., 1983).

Sensory processing is significantly affected during MS as a result of axonal demyelination. Although neuronal degeneration is mainly found in demyelinating lesions and correlates with degree of inflammation, there is evidence for immune-mediated damage in the absence of directly co-localised active demyelinating process (DeLuca et al., 2006). Analysis of sensory neuropeptides in previous studies showed that CGRP expression is increased in the epidermal nerve fibres and keratinocytes of Theiler's Murine Encephalomyelitis Virus MS model. Theiler's virus induces in susceptible mice, an acute disease resembling encephalomyelitis followed by chronic demyelinating disease (Oleszak et al., 2004). In contrast, evaluation of CGRP expression in the dorsal horn of MOG₃₅₋₅₅ induced EAE did not reveal any significant changes in expression between the EAE animals and CFA-treated controls (Olechowski et al., 2009).

Microglia, referred to as macrophages of the CNS represent 12% of the cells present in the CNS. Their functions include phagocytosis, antigen presentation and production of cytokines. Microglia also produce excitatory amino acids such as glutamate, oxidative radicals and nitric oxide (Banati et al., 1993). Treatments that target glial cell metabolism and inhibit activation and proliferation of astrocytes and microglia have proven effective at preventing, or reversing neuropathic pain

behaviour in animal models of spinal cord and peripheral nerve injury (Hains and Waxman, 2006). Three states of microglia have been identified based on development and pathology (Luo and Chen, 2012); first, the resting and ramified microglia present in the healthy CNS. Secondly, the activated, non-phagocytic microglia found in areas involved in CNS inflammation and thirdly, the reactive, phagocytic microglia observed in areas of trauma and infection (Banati et al., 1993). In EAE, changes in glial cell reactivity within the superficial dorsal horn of the spinal cord correlate with the development of cold and tactile allodynia (Olechowski et al., 2009).

Histamine has been implicated in the pathogenesis of both MS and EAE (Jadidi-Niaragh and Mirshafiey, 2010). Upon release from mast cells, it modulates blood brain barrier permeability thereby enhancing leukocyte rolling, adhesion and extravasation of inflammatory cells into the CNS (Abbott, 2000). Tissue levels of histamine correlate with the onset of EAE (Ichigi, 1999) and increased concentrations of histamine in cerebral spinal fluid correlate with relapses in MS patients (Tuomisto L., 1983). Research shows that inhibitors of mast cell degranulation as well as sedating H₁ antagonists modify MS severity and a small study showed that, patients treated with H₁ antagonists remained stable or improved neurologically (Longothesis L.I., 2005). Genetic studies show that histamine and H₁, H₂ and H₃R play important roles in disease progression and EAE susceptibility either by regulating antigen presenting cell (APC) function, the encephalitogenic T cell responses and BBB permeability (Ma, 2002, Musio, 2006).

However the role of the H₄ receptor in autoimmune inflammatory demyelinating disease of the CNS is yet to be elucidated. To assess the role of H₄ receptor signalling in the regulation of autoimmune inflammatory demyelinating disease of the CNS, we studied the effect of the selective H₄ receptor antagonist JNJ 7777120 on MOG₃₅₋₅₅ induced EAE severity. This chapter will look at the effects of JNJ 7777120 treatment on the expression of the H₄R on lymphocytes infiltration into the spinal cord, T cell, microglial, neuronal expression and correlation to disease severity.

5.2 Methods

This chapter summarizes a collaborative study performed in collaboration with Professor Beatrice Passani (University of Florence), including an STSM (COST Action BM0806) spent in Florence during the PhD period.

5.2.1 Experimental animals

All animal manipulations were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the Università di Firenze (I). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 38 Female C57BL/6 mice were used for immunization experiments. Mice were randomly assigned to standard cages, with four to five animals per cage, and kept at standard housing conditions with a light/dark cycle of 12 hrs (8.00-20.00) and free access to food and water.

A total of sixteen 6-8 week old female C57BL/6J mice were used weighing 16-20 g. The mice were purchased from the Jackson Laboratory (Bar harbour) and were housed in standard laboratory cages and had free access to food and water throughout the study period. The experimental procedures were under the guidelines of the Animal Care and Use Committees of the University of Florence (Italy). These sets of procedures were performed in Professor Passani's laboratory

5.2.2 Induction and evaluation of EAE

These sets of procedures were performed by Professor Passani's laboratory.

C57BL/6 female mice, 6–8 weeks of age were obtained from Harlan, Italy, Srl. (Milan, Italy). The mice were housed in macrolon cages on a 12 hour light/dark cycle at 23 °C room temperature (RT), with *ad libitum* access to food and water. Adequate measures were taken to minimize pain or discomfort. Mice were immunized subcutaneously (s.c.) in the flanks and at the base of the tail with a total of 200 µg of MOG_{35–55} (synthesized by EspiKem Srl., Università di Firenze, Italy) per animal

emulsified in complete Freund adjuvant (Sigma, Milan, Italy) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Detroit, Michigan, USA). Immediately thereafter, and again 48 hours later, the mice received an intraperitoneal (i.p.) injection of 500 ng pertussis toxin (Sigma, Milan, Italy) in 100 µl phosphate buffer saline (PBS). The animals were examined daily for weight loss and disability, and were clinically graded by investigators blind to group identity, as follows: zero indicates no signs; 0.5, partial loss of tail tonicity; 1, paralyzed tail; 2, ataxia and difficulty in righting; 3, paralysis of the hind limbs and/or paresis of the forelimbs; 4, tetraparalysis; 5, moribund or death.

5.2.3 Pharmacological treatments

The H₄ receptor antagonist JNJ 7777120 (Johnson & Johnson, San Diego USA) was dissolved in 1% ethanol in physiological saline to give the final dose of 10 mg/kg in 100 µl per mouse. JNJ 7777120 or vehicle was administered by daily intraperitoneal (i.p.) injections for 10 days commencing at D10 post-immunisation and observations for pathology were made for the entire duration of the disease. Mice were randomly assigned to 2 different experimental groups. The mice received daily injections of either JNJ 7777120 or vehicle beginning at D10 p.i. and were sacrificed at D28 p.i.

5.2.4 Clinical grading of mice with classical EAE

These sets of procedures were performed in Professor Passani's laboratory.

The clinical severity of classic EAE is typically scored using a grading scale of 0-5 outlined in table 5.1. Despite the use of genetically identical mice, variability in day of onset and severity of EAE among individual animals in the same experimental group has been observed. The reasons for this variability are unknown. The disease course in EAE experiments is reported in literature by depicting the mean clinical score of an entire group over time (Stromnes and Goverman, 2006).

Grade	Clinical sign	Observation
0	No clinical signs	Extended hind legs; tail extends up and moves, wraps around cylindrical object; normal gait
1.5	Partially limp tail	Extended hind legs; tip of tail droops, does not wrap around cylindrical object; normal gait
1	Paralysed tail	Extended hind legs; tail droops, does not wrap around cylindrical object; normal gait
2	Loss of coordinated movement; hind limb paresis	Hind legs contract when held at the base of the tail; uncoordinated movement; hind limbs reflex when toes are pinched; limp tail
2.5	One hind limb paralysed	Mouse drags one hind limb; one hind limbs does not respond to tail pinch; limp tail
3	Both hind limbs paralysed	Mouse drags both hind limbs; both hind limbs do not respond to toe pinch; limp tail
3.5	Hind limbs paralysed; weakness in forelimbs	Mouse drags hind limbs with difficulty using forelimbs to pull body; forelimbs respond to toe pinch; limp tail
4	Forelimbs paralysed	Mouse cannot move; forelimbs do not respond to toe pinch, limp tail
5	Moribund	No movement; cold to the touch, altered breathing

Table 5.1 Outline of the guidelines used to grade the pathological progression of clinical EAE. Adapted from (Stromnes and Goverman, 2006).

5.2.5 Determination of serum antibodies by enzyme-linked immunosorbent assay (ELISA)

Serum was obtained from three animals in each treatment group at D28 p.i. five serum dilutions ranging from 1:50 to 1:2500 were distributed onto polystyrene ELISA plates pre-coated with MOG₃₅₋₅₅ peptide in order to obtain a titration curve. Serum from non-immunised mice age and gender matched was distributed as control samples. Bound antibodies were revealed with goat anti-mouse IgM+IgG+IgA (H+L) immunoglobulin conjugated to alkaline phosphatase and p-nitrophenyl phosphate as substrate. Absorbance was measured at 405 nm. Results are reported as optical density (O.D)

5.2.6 Cell isolation

These sets of procedures were performed in Professor Passani's laboratory.

Cells were isolated from lymph nodes, spleen and spinal cord and analysed for proliferative response and phenotype as previously described (Gourdain et al., 2012). Lymphocytes from MOG₃₅₋₅₅-immunized mice treated or not were cultured in complete Rosewell Park Memorial Institute media (RPMI) in 96 wells plates (2×10^5 cells/well) and stimulated with antigen at day 3, and the proliferative response was measured by [³H] thymidine incorporation test after 48 hours as described above (Luccarini et al., 2008). To determine mononuclear cell phenotype, surface markers have been evaluated by means of anti-mouse conjugated monoclonal antibodies [CD4 (AlexaFluor 488), IFN- γ (PerCP-Cy5.5), anti-rabbit H₄ receptor antibodies (Lethbridge and Chazot, 2010a), revealed with anti-rabbit Alexafluor-488 secondary antibodies/Cy3 secondary antibodies, CD11c (PerCpCy5), CD11b(PE), CD3 (PE) from AbCam (Cambridge, UK), BioLegend and eBioscience (both San Diego CA, USA), respectively. Cells were analyzed on a four-color Epics XL cytometer (Expo32 software; Beckman Coulter). Cell viability was tested by means of propidium iodide or 7-actinomycin staining (Molecular Probes). Staining was performed in PBS 1% FCS for 25 min at 4°C followed by two washes. Later on, cells were analyzed by flow cytometry.

5.2.7 Cytokine determination assay

This procedure was performed in Professor Beatrice Passani at the University of Florence.

Cells isolated from the draining lymph nodes at D18 p.i were cultured at 1×10^6 cells/well in a 48-well plate with phytohemagglutinin (PHA, 5 $\mu\text{g}/\text{ml}$, SIGMA Aldrich) or MOG_{35–55} (2 $\mu\text{g}/\text{ml}$, EspiKem Srl., Università' di Firenze, Italy); supernatants were collected after 48 hours and evaluated by ELISA for cytokine contents (IFN- γ , IL-6 and IL-10). For IL-4 determination, samples were collected after 5 days. All ELISAs were performed with ELISA Ready-SET-Go! (eBioscience)

5.2.8 Histology

This procedure was performed in Professor Beatrice Passani at the University of Florence.

At the time of sacrifice, the mice were anesthetized with pentobarbital (65 mg/kg, i.p.) The spinal cord was removed from the column and fixed in 4% (v/v) paraformaldehyde in PBS for 48 hours. The spinal cord samples were dehydrated through a series of ethanol changes from 70%, 80%, 95% and 100% with 2 changes at each concentration and left in the ethanol for 1 hour each time. This was followed by 3 xylene changes of 1 hour each time and then 2 paraffin wax changes of 1.5 hours at 56–58°C. The final step was embedding the tissue in molten paraffin molds until the paraffin solidified.

5 μm slices were cut using a microtome and then placed in a heated water bath to unravel the slices. These were removed and mounted onto glass slides. The sections were dried overnight at 37°C as lower baking temperatures are better for subsequent antibody detection. Once dry, the glass slides were put in a slide box and stored at room temperature. To rehydrate the paraffin sections for subsequent methods (paraffin to water), the sections were put through 2, 3 minute changes of xylene followed by a descending series of ethanol changes (i.e. 100%, 90%, 70%) for

3 minutes each and then rinsed in distilled water for 5 minutes. Serial sections were stained with haematoxylin and eosin (H&E), Luxol Fast Blue (LFB)-cresyl violet

5.2.9 Immunofluorescence

Following rehydration, the glass mounted paraffin embedded sections were treated with 10% (v/v) methanol and 3% (v/v) hydrogen peroxide in 50 mM TBS, pH 7.4 for 30 minutes to quench endogenous peroxidase activity. The sections were then given 3, 5 minute washes in 0.2% triton x-100 (v/v) TBS solution. Sections were incubated in TBS, 0.2% (w/v) glycine to mop up residual unreacted aldehyde groups from the fixative. Non-specific binding sites in the tissue were blocked by incubating with donkey/horse serum (10% v/v) in TBS/triton x-100 (0.2% v/v/) for 60 minutes at room temperature. Sections were then incubated overnight at 4°C in anti-mouse H₄ antibody at a final concentration of 1 µg/ml in donkey serum/TBS (1% serum, v/v). Following overnight incubation, the sections were given 3 5 minute washes in TBS/triton x-100 (0.2% v/v/) before a 2 hour incubation in a fluorescent secondary antibody (1:500 dilution) in 1% serum and TBS pH7.4. After the second incubation the slides were given 3, 5 minute washes in TBS/triton x-100 (0.2% v/v/), left to air dry in the dark, mounted with DPX mounting solution and stored in the freezer before or after microscopy. The majority of these studies were performed in the University of Durham.

5.2.10 Data analysis and statistics

Statistical analyses were performed using GraphPad Prism version 5 (San Diego, CA, USA). Mean and SEM were calculated for illustration in figures. For each animal, the onset day was recorded as the day post immunization (D) when the first clinical manifestations appeared (score > 0). Differences between groups were tested by Mann–Whitney test, for their score course. Significant differences between controls and JNJ 7777120 treated-mice were established using the Student's *t*-test for the day of onset and of maximum score. Comparisons of parameters such as cytokines, cell proliferation and phenotypes, MOG antibodies were performed using the unpaired

Student's *t*-test. In all cases, *p* values less than 0.05 were considered statistically significant.

5.3 Results

5.3.1 Exacerbation of Experimental Autoimmune Encephalomyelitis in mice treated with JNJ 7777120 compared to saline treated controls.

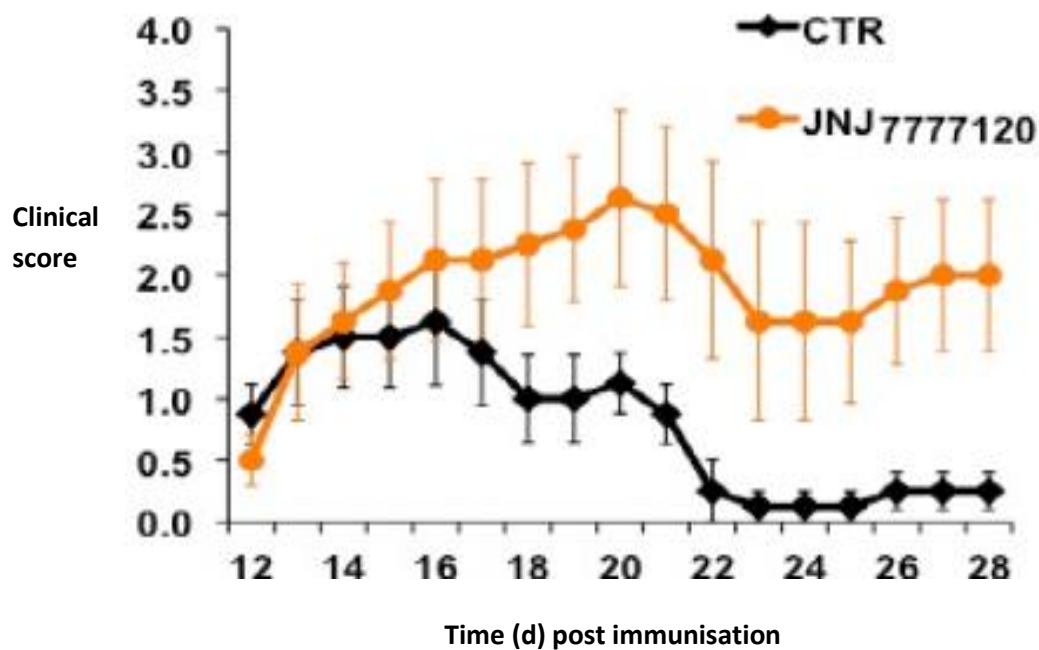


Fig 5.1 Clinical progression of disease was observed in the saline and JNJ 7777120 treated mice.

Increase in the clinical score is proportional with a worse pathological state in the EAE mice. Disease onset was similar in the two groups with symptoms of EAE becoming apparent approximately D11 p.i. Clinical scores begun diverging at D15 p.i. after initial onset of disease EAE mice treated with JNJ 7777120 gave a higher clinical score, compared to the saline treated EAE controls. Data is according to maximal clinical score achieved at any time during the experiment. $P < 0.05$ by Mann-Whitney ($n = 6-8/\text{group}$).

5.3.2 Immunohistochemical assessment of spinal cord pathology in the saline and JNJ 777120 treated EAE mice

The level of inflammatory infiltrates was assessed immunohistochemically through the use of haematoxylin and eosin.

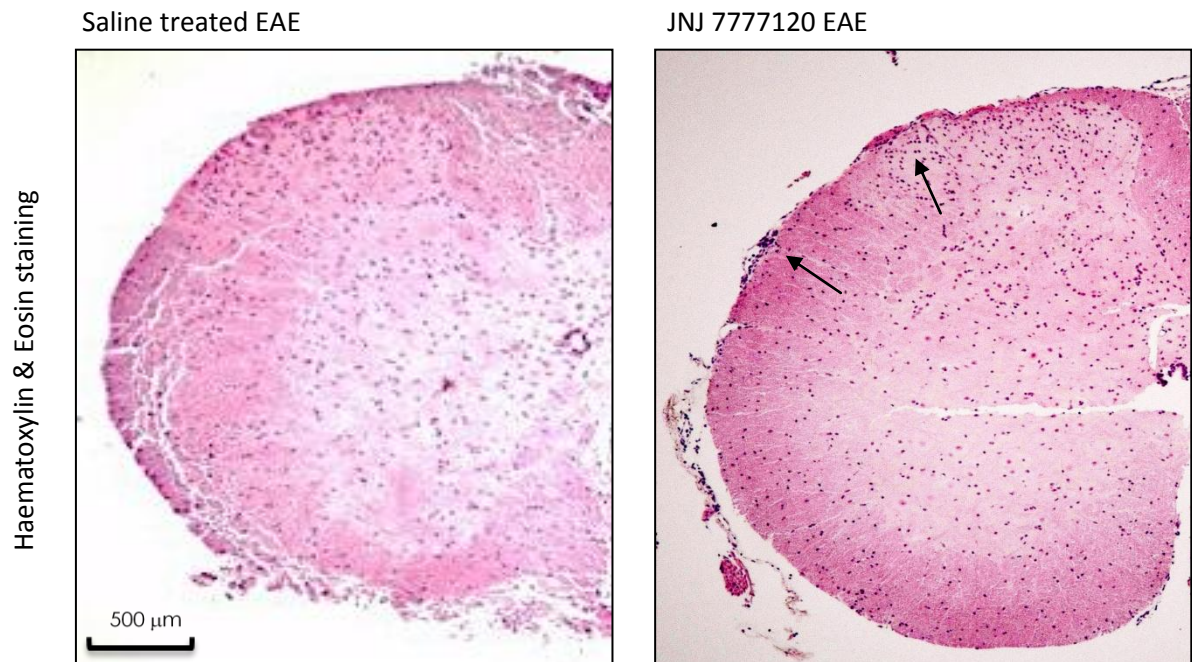


Fig 5.2a EAE was induced in C57/Bl6 mice with MOG₃₅₋₅₅ (100 µg s.c. and PTX 500 ng i.v.) on days 1 and 3.

The two panels show lumbar spinal cord sections stained with haematoxylin and eosin. This shows infiltration of autoreactive immune cells within the spinal cord. JNJ 777120 treated mice had visually more infiltrates than saline-treated control mice with the arrows pointing to clusters of infiltrating immune cells. This is representative data from three experiments.

Demyelination was observed through the use of luxol fast blue staining

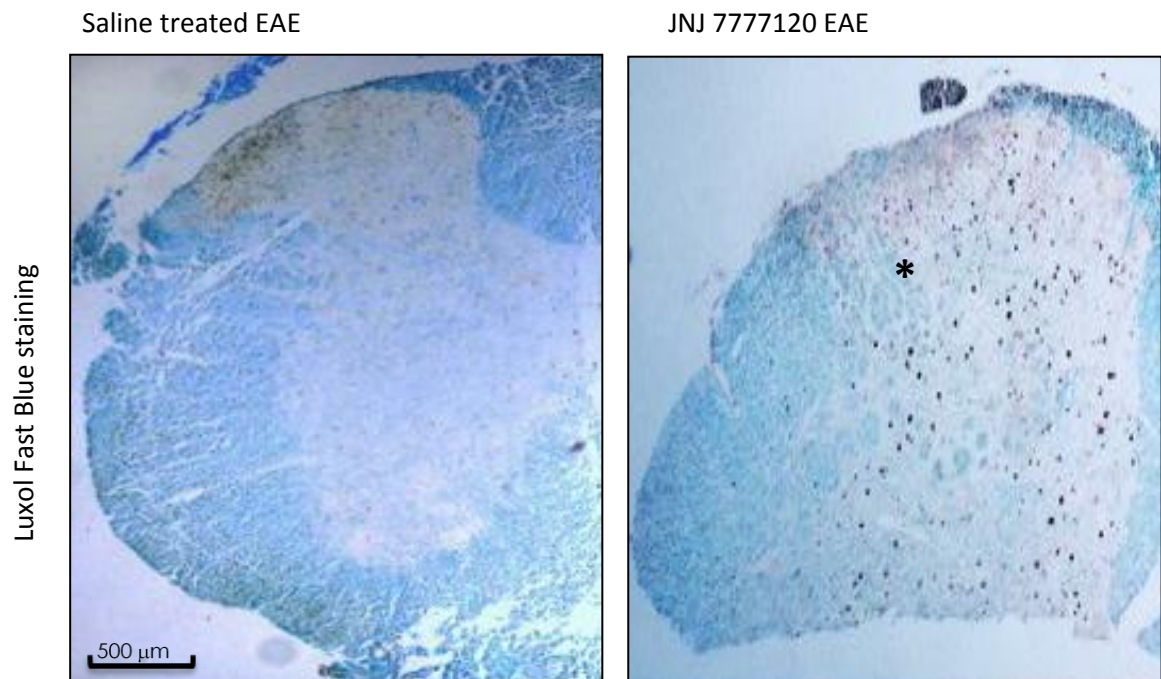


Fig 5.2b EAE was induced in C57/Bl6 mice with MOG₃₅₋₅₅ (100 μ g s.c. and PTX 500 ng i.v.) on days 1 and 3.

Luxol fast blue (LFB) staining was used to visually assess the degree of myelin loss in the lumbar sections of the spinal cord. LFB staining revealed larger plaques of demyelination at the sites of inflammatory infiltrates in the spinal cord of JNJ 7777120, compared to saline treated EAE mice show more clusters of inflammatory infiltrates in the dorsal spinal cord compared to the untreated EAE controls [asterix]. This is representative data from three experiments.

5.3.3 Evaluation of astrocyte activation in the lumbar spinal cord of JNJ 777120 treated EAE and saline treated control mice

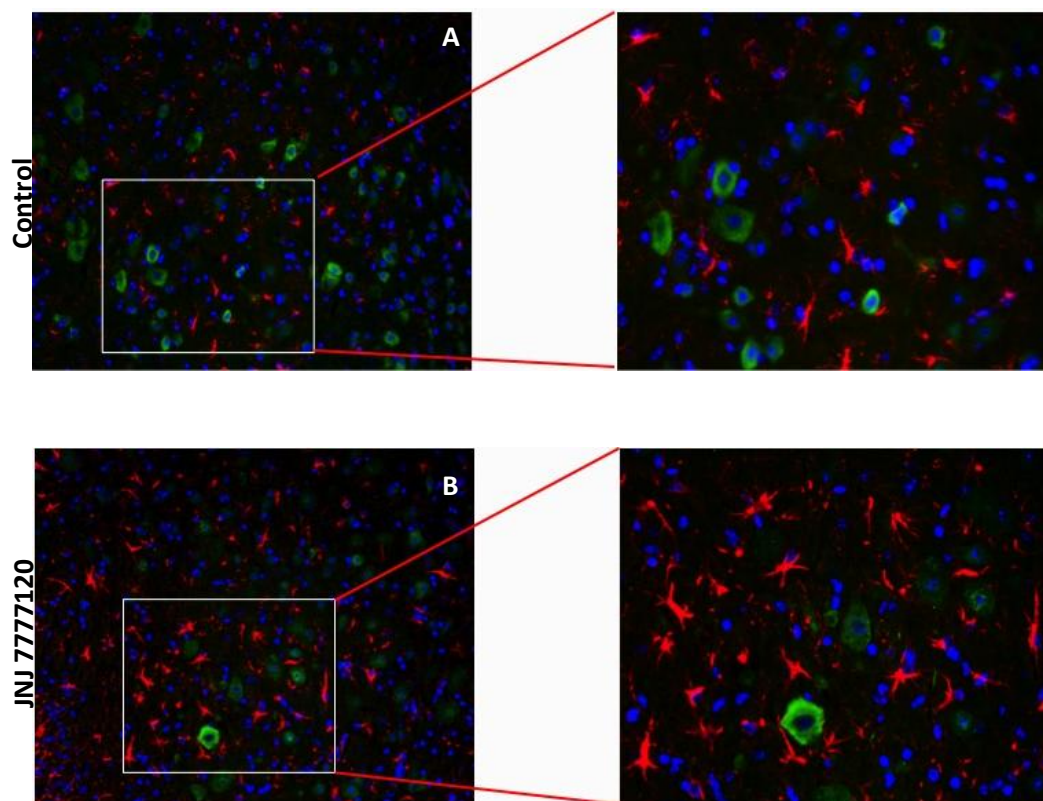


Fig 5.3 Immunofluorescence labelling of the lumbar spinal cord from saline and JNJ 777120 treated EAE mice. The lumbar spinal cord sections have been labelled with the anti-GFAP antibodies (red) an intermediate filament protein expressed in astrocytes and NeuN (green) a marker for neurons. The nucleus has been stained with DAPI (blue). This is representative data from three experiments.

In the saline treated EAE mice, a small number of hypertrophic astrocytes were observed. In JNJ 777120 treated mice, an intense glial activation with cells stained for GFAP with enlarged cells bodies and long processes was observed. The results show an increased presence of activated astrocytes with outwardly extending processes in the in JNJ 777120 treated EAE mice **[B]** compared to the saline treated EAE control mice **[A]**.

5.3.4 Effect of the H₄R antagonist JNJ 7777120 on microglia activation in the spinal cord of EAE mice

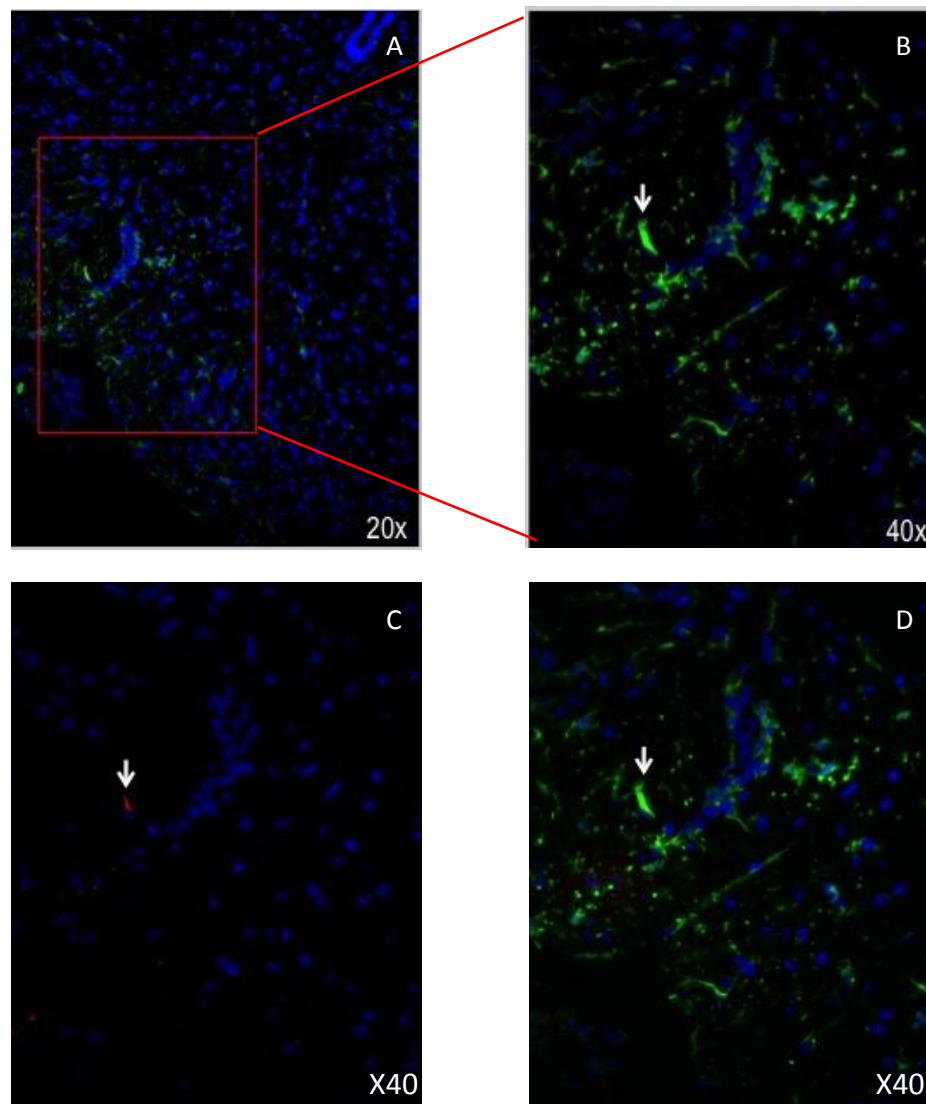


Fig 5.4 Immunofluorescence labelling of the lumbar spinal cord from saline treated EAE controls.

A shows a magnified section of the ventral median fissure of the lumbar spinal cord. **B** sections were double labelled with anti-Iba1 antibodies (green) to detect macrophages and microglia, and **C** anti-IFN γ antibodies (red) and counterstained with DAPI (blue). Sections were visualised with 20x and 40x lenses. **D** superimposed image of B and C. This is representative data from at least three experiments.

The presence of IFN γ + activated microglia in the spinal cord of the saline treated control EAE mice is undetectable, as represented by the lack of staining.

5.3.5 Effect of the H₄R antagonist JNJ 7777120 on microglia activation in the spinal cord of EAE mice

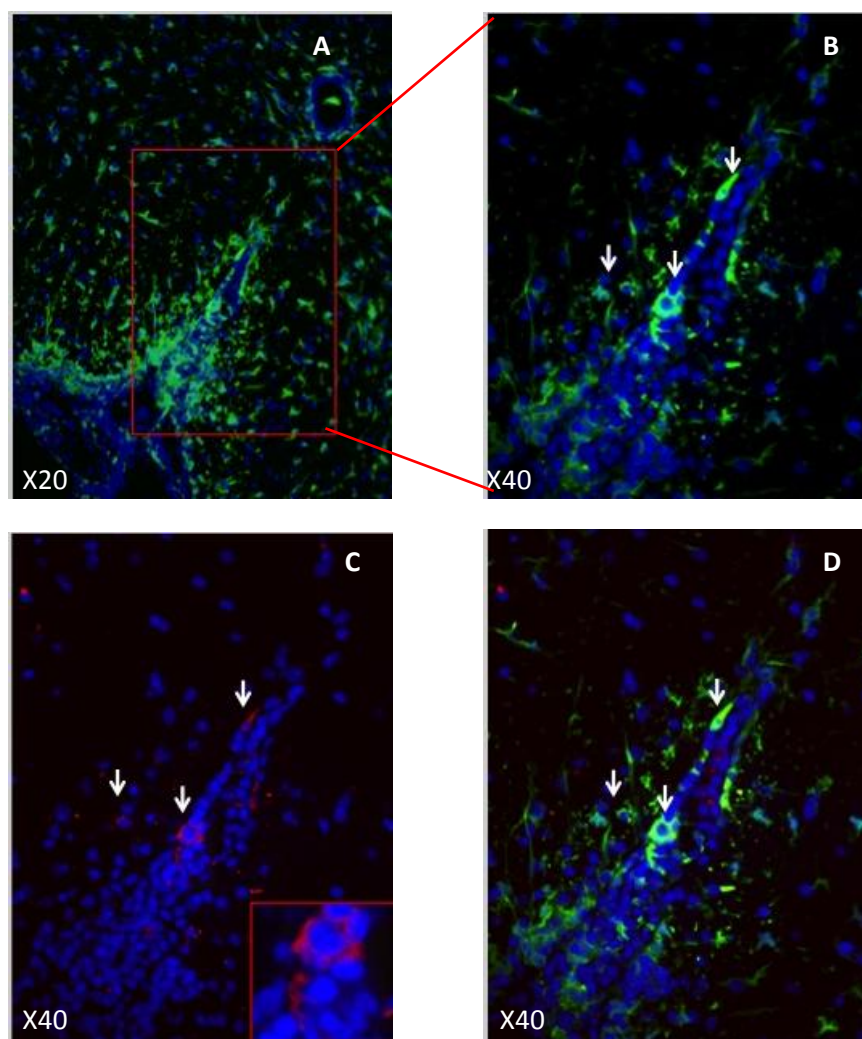


Fig 5.5 Effect of JNJ 7777120 on microglia activation in the spinal cord of EAE mice

A shows a magnified section of the ventral median fissure of the lumbar spinal cord. **B** sections were double labelled with anti-Iba1 antibodies (green) to detect macrophages and microglia, and **C** anti-IFN γ antibodies (red) and counterstained with DAPI (blue). Sections were visualised with 20x and 40x lenses. **D** superimposed image of B and C. All experiments were performed on mice D28 p.i. with comparable scores (<2.0). This is representative data from three experiments.

The results show increased microglia activation through intense staining with Iba1, which is up-regulated during activation of macrophages and microglia. IFN γ ⁺ staining is representative of activated macrophages. There is increased macrophage and microglia activation in JNJ 7777120 treated mice compared to the saline treated EAE controls.

5.3.6 Expression of calcitonin gene-related peptide in the lumbar spinal cord of the saline and JNJ 777120 treated EAE mice.

Expression of the neuropeptide CGRP between the saline and JNJ 777120 treated EAE mice was assessed immunohistochemically through the use of the antibody for CGRP.

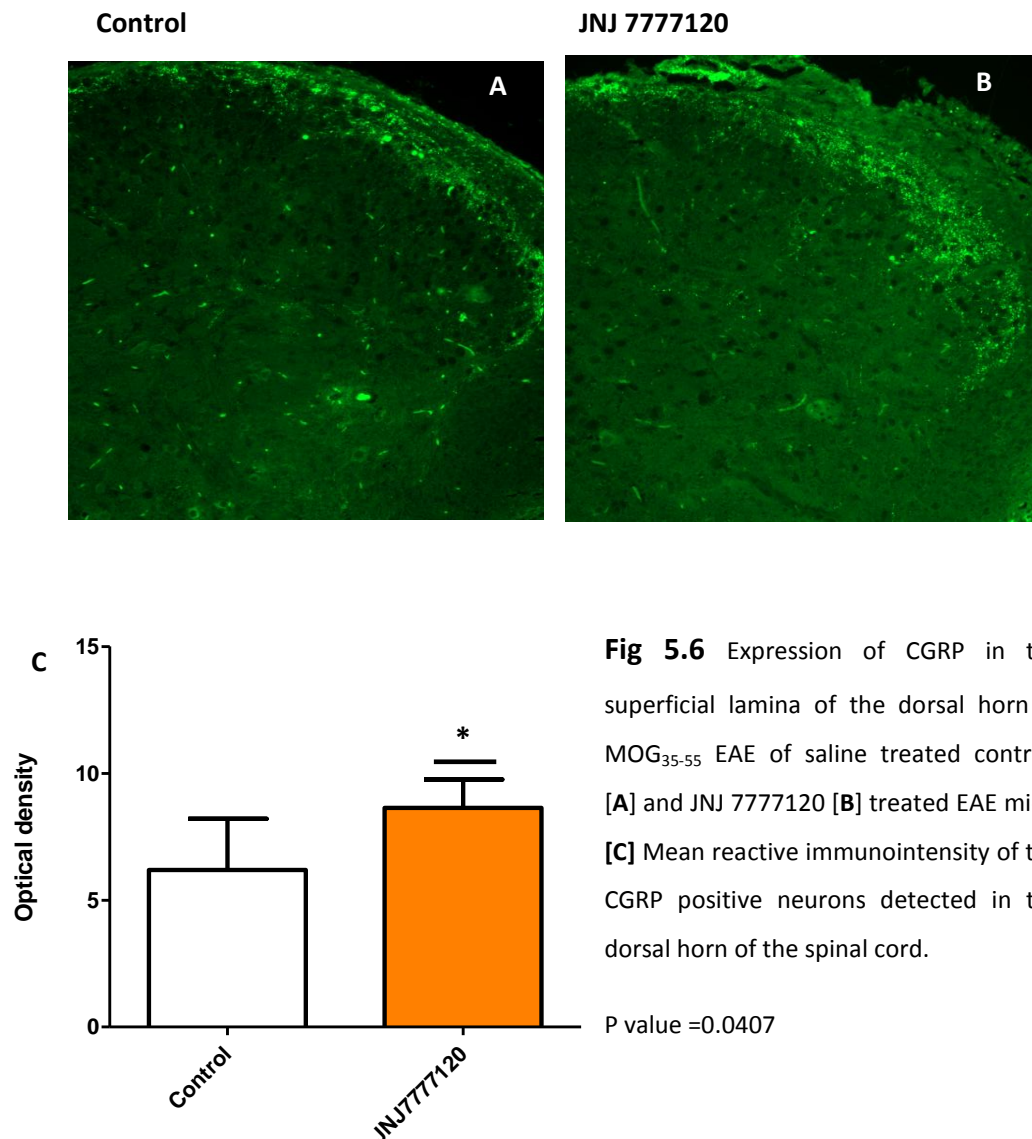


Figure 5.6 shows lumbar spinal cord sections stained with anti-CGRP antibodies saline treated (A) and JNJ 777120 treated (B) EAE animals taken from the acute phase of the disease. CGRP is significantly upregulated in the superficial lamina of the JNJ 777120 treated EAE mice [Control n=4, JNJ 777120 n=4].

The optical density of immunoreactivity was examined specifically in a region that encompassed the superficial lamina (I-II) of the dorsal horn. The level of background staining was determined for each section and subtracted for all optical density measurements using ImageJ software. Statistical analysis was carried out using GraphPad Prism, using the Student's t-test and significance was set at $p \leq 0.05$.

5.3.7 Effect of JNJ 777120 on *in vitro* anti-MOG₃₅₋₅₅ serum concentration

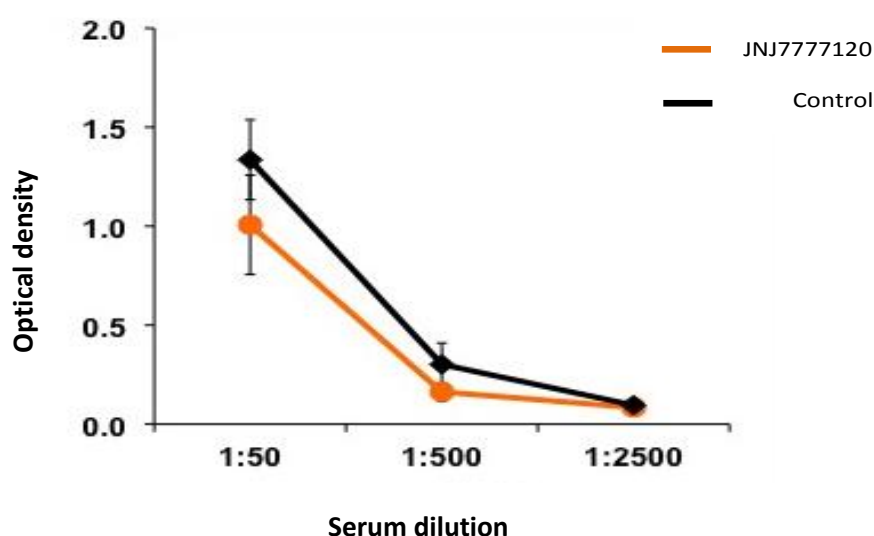


Fig 5.7 Anti-MOG₃₅₋₅₅ concentrations were measured in the serum of EAE D28 days p.i. JNJ 777120 was injected i.p. daily at a dose of 10 mg/kg. Controls received an equivalent volume (100 μ l) of saline. (n=3 per group)

Anti-MOG₃₅₋₅₅ titration curves were comparable between the control and JNJ 777120 treat EAE mice. This signifies that there were no differences in antibody production in the sera of the EAE mice.

5.3.8 Effect of JNJ 777120 on the *in vitro* proliferative response of lymphocytes and splenocytes from EAE mice, in the presence of MOG₃₅₋₅₅

Lymphocytes [A] and splenocytes [B] were isolated, plated at 2×10^5 cells/well and incubated with MOG₃₅₋₅₅ at the indicated concentrations for 72 hours in the presence of ³[H] Thymidine for 12 hours. (n=3 per group)

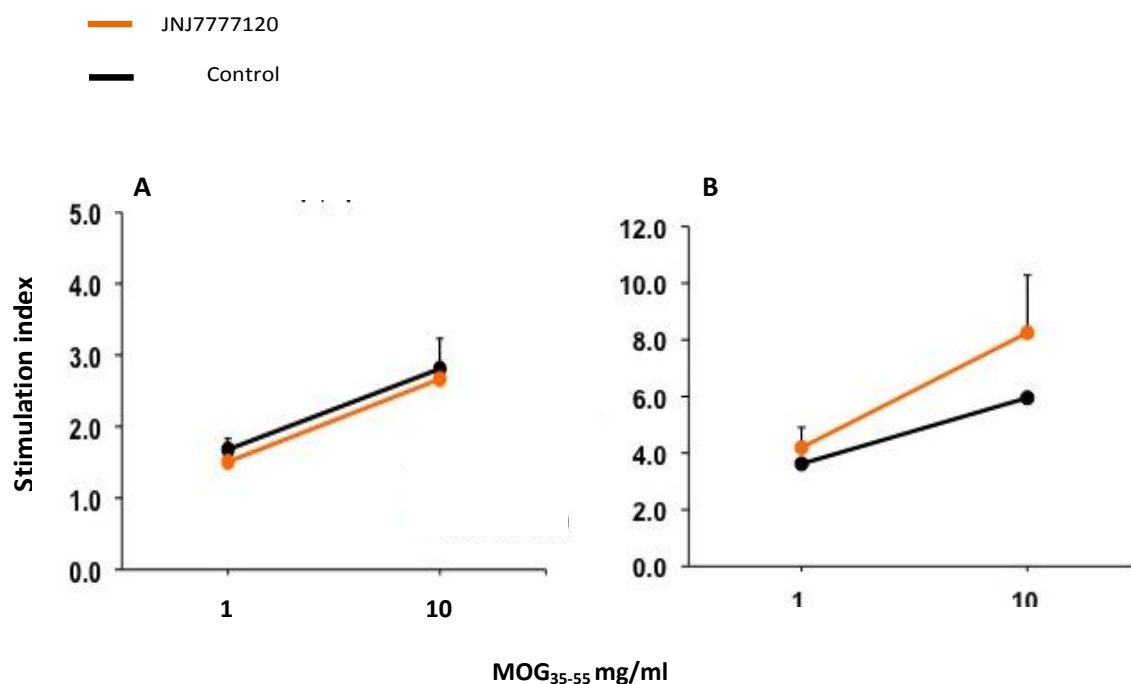


Fig. 5.8 Proliferative response of lymphocytes [A] and splenocytes [B] in the presence of MOG₃₅₋₅₅ peptide

In vitro proliferation of lymphocytes and splenocytes isolated at D28 p.i. were incubated for 72 hours with two doses of MOG₃₅₋₅₅. Incorporation of ³[H] Thymidine was used to measure the rate of lymphocyte and splenocyte T cell proliferation during the last 12 hours of culture.

There was no significant difference in lymphocyte proliferation between isolates from untreated EAE and JNJ 777120 treated mice in the presence of MOG₃₅₋₅₅ at the indicated concentrations. Therefore, the differences in disease score were not due to differences in T cell proliferative responses against the immunising antigen or T cell antigen-presenting cell migration in lymph nodes of the EAE mice.

Cell proliferation statistical analysis was carried out using GraphPad Prism, using the Student's t-test and significance was set at $p \leq 0.005$.

5.3.9 Effect of JNJ 777120 on the *in vitro* cytokine concentrations in the lymph nodes of MOG₃₅₋₅₅ immunised mice

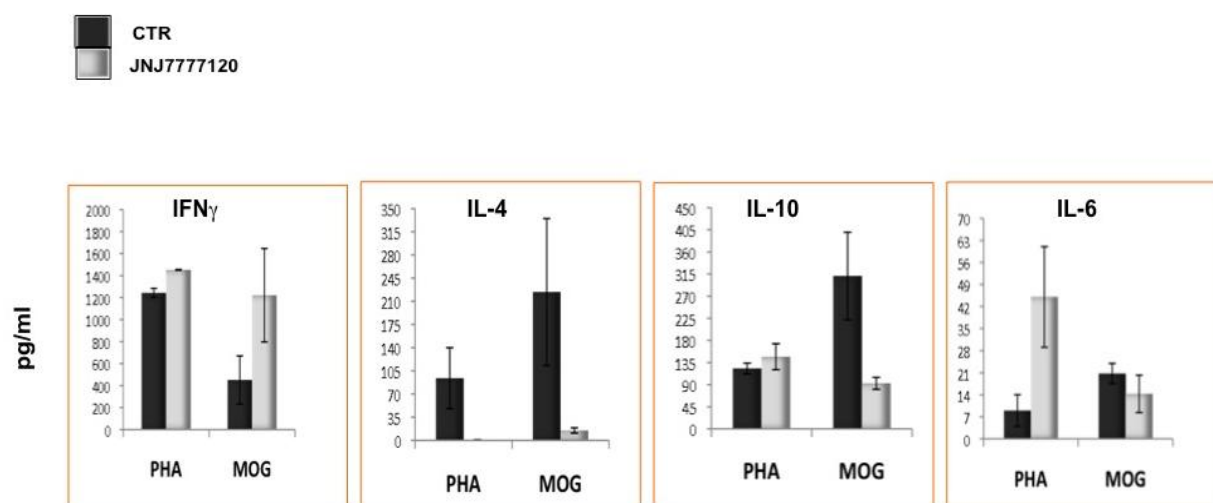


Fig. 5.9 Cytokine production in the lymph nodes in response to MOG₃₅₋₅₅ peptide and phytohaemagglutinin (PHA).

JNJ 777120 treatment significantly increased IFN γ ($p=0.01$) release in the lymph node cells compared to controls, where as significantly less IL-4 (0.042) was released in the supernatants of LN cells. No significant differences were observed in IL-6 and IL-10 production ($p=0.13$).

ELISA determination of IFN γ , IL-6, IL-10 and IL-4 in the supernatants of Lymph node cells isolated at D18 p.i. Cells were cultured at 2.5 million cells /well in 24 well plates for 72 hours in the presence of MOG₃₅₋₅₅ (50 $\mu\text{g/ml}$) or Phytohaemagglutinin (PHA) (5 $\mu\text{g/ml}$) and then tested for the presence of IFN γ , IL-4, IL-10 and IL-6. ($n=3$ for control, $n=5$ for JNJ 777120 treated mice).

Analysis of *in vitro* cytokine concentrations in the lymph node cells was performed using the unpaired Student's t-test, where $P \leq 0.05$ was considered statistically significant.

5.3.10 Effect of JNJ 777120 on the *in vitro* cytokine concentrations in the splenocytes of MOG₃₅₋₅₅ immunised mice

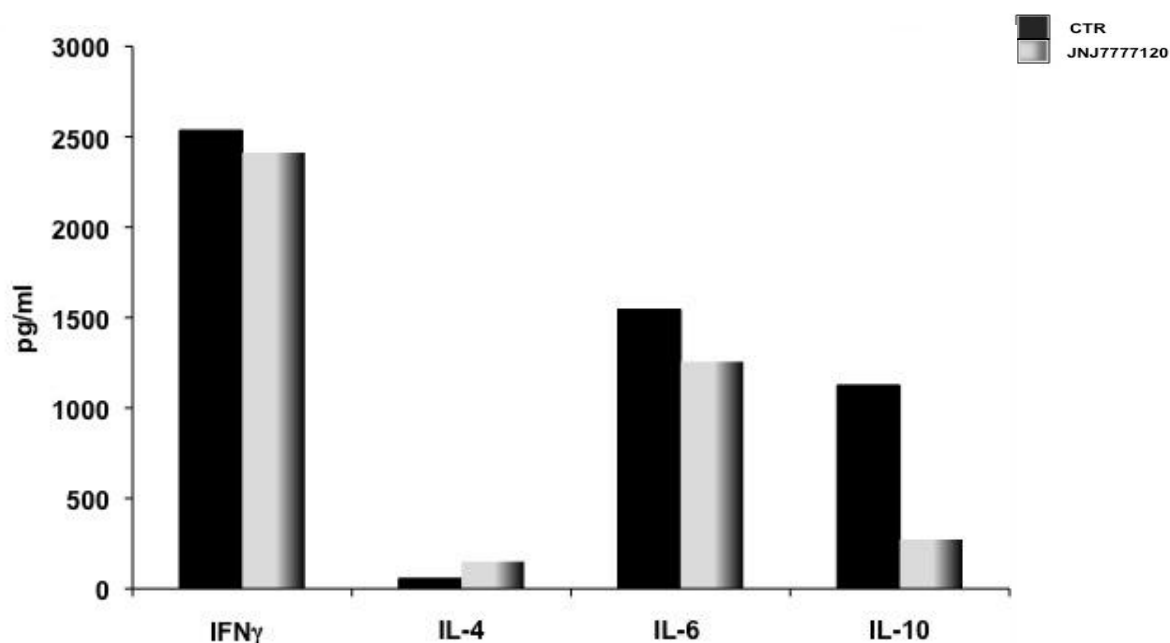


Fig 5.10 Cytokine production in the splenocytes in response to MOG₃₅₋₅₅ peptide

IFN γ and IL-6 concentrations were equivalent in JNJ 777120 treated and saline treated EAE control mice. IL-4 concentrations were low in both treatment groups. However JNJ 777120 treatment reduced production of IL-10.

Splenocytes from EAE controls and JNJ 777120 treated EAE mice were cultured at 2.5 million/well in a 24 well plates for 72 hours in the presence of MOG₃₅₋₅₅ (50 μ g/ml). Supernatant were collected after 72 hours and tested by ELISA for the presence of cytokines. (n=3 per group).

Analysis of *in vitro* cytokine concentrations in the splenocytes was performed using the unpaired Student's t-test where p values ≤ 0.05 were considered statistically significant.

5.3.11 Effect of JNJ 777120 on *in vivo* profile of CD4⁺ T lymphocytes infiltrating the spinal cord of EAE mice

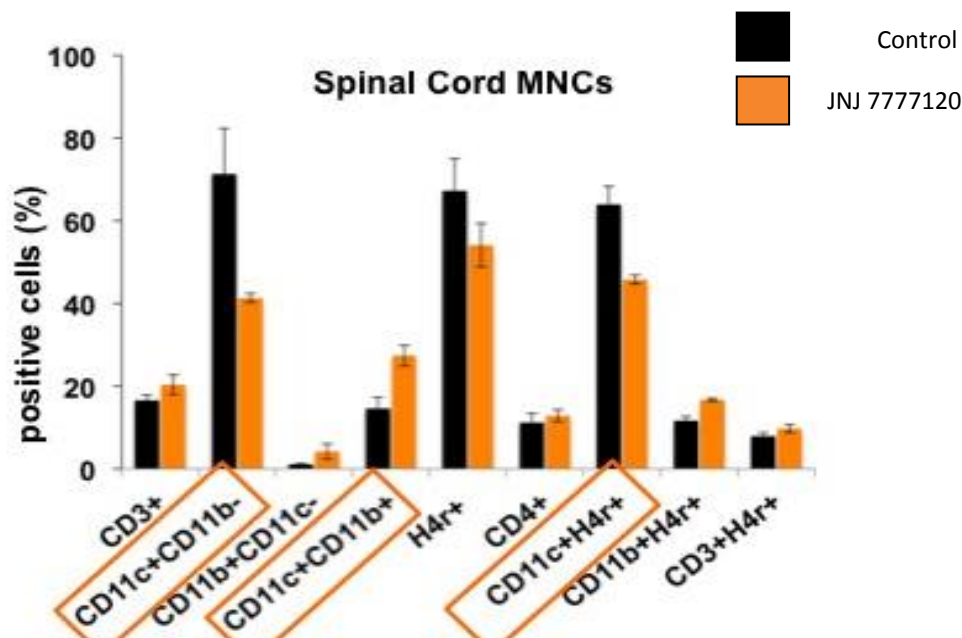


Fig 5.11 The frequency of T cell mononucleocytes (MNC) was determined in the spinal cord of the saline and JNJ 777120 treated mice

Analysis of T lymphocytes infiltrating the spinal cord of the EAE mice revealed the presence of H₄ receptor positive cells, CD11c dendritic cells and macrophages. An increase in CD4⁺ H₄R⁺ T cells in JNJ 777120 treated mice was observed, although not statistically significant. Comparisons of parameters between T cell frequencies were performed using the unpaired Student's t-test. In all cases, *p* values ≤0.05 were considered statistically significant.

5.4 Discussion

Histamine has been implicated in the pathogenesis of inflammatory autoimmune diseases of the CNS such as MS and its animal model, EAE (Jadidi-Niaragh and Mirshafiey, 2010) and the H₁, H₂ and H₃ receptors have been shown to be involved in susceptibility to EAE. The recently discovered H₄ receptor is mainly expressed on haematopoietic cells and is postulated to have an immunomodulatory function during allergic conditions and inflammation (Thurmond et al., 2008b). Clinical symptoms of MS include sensory disturbances, optic neuritis, diplopia, limb weakness, clumsiness, gait ataxia, neurogenic bladder, bowel symptom and also pain (Svendsen et al., 2005). Pain in MS can be ascribed to various conditions including musculoskeletal pain, tonic spasms and chronic neuropathic pain that results from sclerotic plaque lesions affecting sensory pathways (Truini et al., 2012). So far, the mechanisms underlying pain in MS remain unclear and its treatment remains difficult.

EAE is used extensively as an animal model of human demyelinating disease and is induced by subcutaneous immunisation with myelin antigens emulsified in adjuvant or adoptive transfer of encephalitogenic T cells into susceptible animals, mainly mice (Stromnes and Goverman, 2006). Histamine and mast cell degranulating agents that cause its release have been shown to alter the permeability of the BBB. First generation antihistamines with the ability to cross the BBB are associated with reduced MS risk. For example, patients treated with the H₁ receptor antagonist hydroxyzine were shown to remain stable or display improved neurological condition (Logothetis et al., 2005) and was shown to inhibit EAE and activation of brain mast cells (Dimitriadou et al., 2000). In the EAE model, increased histamine levels within the cerebral spinal fluid correlate with onset of disease and severity, which are reduced by H₁ receptor antagonists (Orr and Stanley, 1989). The day of onset of clinical signs varies depending on the model but is usually between days 9 and 14 days post-immunisation. The clinical features of acute fatal EAE in the commonly studied species include tail weakness and paralysis followed by hind limb weakness and sometimes paralysis. Forelimb weakness occasionally occurs (Stromnes and Goverman, 2006).

In our model, the severity of the MOG₃₅₋₅₅-induced EAE was assessed daily using a previously validated scale (Stromnes et al., 2004). The clinical symptoms of EAE in the mice manifested at day 10 and peaked at day 16 post-immunisation in the saline treated EAE control mice; whereas the clinical severity peaked 20 days post-immunisation in the JNJ 7777120 treated EAE mice. JNJ 7777120 has been used effectively in a variety of animal models of inflammatory disease (Coruzzi et al., 2007, Coruzzi et al., 2012) and has been the reference antagonist of choice to study the H₄ receptor. Although it has a reasonable bioavailability (30%) (Thurmond et al., 2004a), this compound has a short *in vivo* half-life and therefore, during the course of this study the assumption was made that repeated dosing was needed to maintain a therapeutically effective plasma concentration. In contrast to the hypothesis, JNJ 7777120, an antagonist at the human H₄ receptor appeared to exert agonist properties at the mouse H₄ receptor by increasing and extending the severity of the disease. JNJ 7777120 treated mice displayed a worse disease phenotype compared to the saline-treated group. However, the clinical score of the saline treated EAE controls began to reduce at 17 days post-immunisation and this is consistent with the rapid neurological recovery and this has been previously reported (Pender, 1989). In contrast, the clinical score of the JNJ 7777120 treated mice only reduced after day 20 and the reason for this could be reduced inflammation of the CNS as a result of cessation of drug administration. JNJ 7777120 was administered to the mice for only 10 days (from day D10-20) and the clinical score is possibly a result of inflammation in the CNS due to drug presence in the spinal cord.

Studies have investigated the sensory properties of hind paws as a confirmation for the presence of hyperalgesia and allodynia, components of MS related pain. Lu *et al.* (Lu et al., 2012) showed that during MOG₃₅₋₅₅ induced EAE, animals develop significant thermal hyperalgesia and mechanical allodynia towards low intensity stimuli during disease onset and peak phase. A relapsing-remitting form of myelin proteolipid protein 131-155 (PLP₁₃₁₋₁₅₅) induced EAE in the SJL mice found that this mice model of EAE develops thermal hyperalgesia in the clinically affected body regions emerging at the peak of neurological deficit (Aicher et al., 2004a). A study by Olechowski *et al.* (2009) showed that there were no observable differences in

sensitivity to noxious heat in the hind paws of a group of MOG₃₅₋₅₅ induced EAE. However, the same cohort indicated the presence of cold allodynia and robust tactile allodynia as shown by increased withdrawal latencies and reduced withdrawal threshold in response to acetone application and Von Frey filaments, respectively. As clinical symptoms worsen during the chronic phase of disease, withdrawal thresholds tend to decrease below those seen in the baseline, suggesting that cold and tactile allodynia are major sensory disturbances detected in MOG₃₅₋₅₅ model of EAE. These symptoms develop prior to the onset of overt neurological symptoms neuropathic pain can be a major symptom just before, or simultaneous with onset of clinical deficits in the disease. On the other hand, this hypersensitivity is independent of the specific extent of neurological deficits that arise during the disease.

The initial aim of this study was to observe the presumed anti-inflammatory effects of the H₄ receptor antagonist JNJ 7777120 and evaluate whether it is a good candidate for preclinical neuropathic inflammatory conditions in a model of MOG₃₅₋₅₅ EAE. Histological assessment of the lumbar spinal cord shows JNJ 7777120 treated EAE mice have more clusters of mononuclear inflammatory infiltrates in the dorsal horn, ventral horn and intermediate regions of grey matter, compared to the saline treated EAE control mice. This is a result of increased permeability of the blood brain barrier in the diseased state, to circulating leukocytes. Following luxol fast blue staining, it was observed that JNJ 7777120 treated animals have increased neuron death and demyelination due to the presence of myelin plaques in the white matter of the lumbar spinal cord and this is an important cause of the neurological signs seen in the animals. These results show the presence of both demyelination and inflammation and are consistent with the higher clinical score in this group of animals and a subsequently worse off clinical/pathological state, as has been reported previously (Tafreshi et al., 2005). MOG₃₅₋₅₅ induced EAE presents with increased Nissl substance in motor neurons, progressive axonal loss due to reduction of nerve fibres per mm² and demyelination is observed simultaneously with symptom development (Kuerten et al., 2011). The ascending progression of weakness and tail nociception in EAE is partly accounted for by the demyelination of the small diameter afferent fibres in the sacrococcygeal DRGs, dorsal roots and

dorsal entry zones as previously described (Pender, 1986, Pender et al., 1991). These results are consistent with previously published data where H₄ receptor has been shown to negatively regulate MOG₃₅₋₅₅ induced EAE severity; mice lacking this receptor display heightened disease and immunopathology coupled with increased BBB permeability during the acute phase (del Rio et al., 2012).

Treatment with JNJ 7777120 has no effect on production of anti-MOG₃₅₋₅₅ serum antibodies, therefore the differences in clinical severity were not a result of antibody production. Dunford *et al.* (2006) showed that blocking H₄ receptors on dendritic cells reduced the numbers of eosinophils and specific IgE antibodies in allergic mice. In light of these results, further immunological assessment was performed. EAE is mediated by inflammatory CD4⁺ T cells with the ability to infiltrate the CNS, activate resident microglia and recruit macrophages. Previous work has shown the peak of pathological disease corresponds to the peak in the number of myelin specific CD4⁺ T cells along with F4/80⁺ macrophages, dendritic cells, microglia and astrocytes (Olechowski et al., 2009). These innate cells lead to the subsequent destruction of the CNS myelin sheath and oligodendrocyte death.

Widespread glial activation is commonly noted in MS and high levels of both astrocytes and microglia/macrophage activation in both control mice and those treated with JNJ 7777120 have been demonstrated. Immunofluorescent staining of spinal cord sections from the lumbar region show increased astrocyte activation. However, H₄ receptor expression was absent on the astrocyte populations of both treatment groups. Astrocyte functions include biochemical support and nutritional supply of the endothelial cells that form the BBB, as well as repair and maintenance of damaged CNS areas. Further studies revealed increased clusters of IFN γ activated microglia infiltration and macrophages in the white matter of the lumbar region of the spinal cord. Activated microglia are efficient antigen presenting cells (Chastain et al., 2011, Aloisi, 2001) and evidence shows that they readily up regulate MHC class II expression in all inflammatory and neurodegenerative disease (Kreutzberg, 1996). The increased macrophage presence, astrocyte and microglial activation observed in the spinal cords of JNJ 7777120 treated EAE mice confirms the presence of advanced inflammation-induced neurodegeneration (Ponomarev et al., 2007). Microglia are

activated earlier than astrocytes and both types of cells are considered to be the principal source of pro-inflammatory (IL-1, TNF α) and immune regulatory (IL-12) cytokines in the CNS (Luo and Chen, 2012). Resident glial cells within the spinal cord are recognised as critical mediators of central sensitisation (Joyce A DeLeoa, 2001) through their release of cytokines. Treatments that target glial cell metabolism and inhibit the activation and proliferation of astrocytes and microglia have proven to be effective at reversing or preventing neuropathic pain behaviours in animal models of spinal cord injury (Hains and Waxman, 2006). Intrathecal administration of antagonists specific for proinflammatory IL-6, TNF and IL-1 block and fully reverse the allodynia following sciatic inflammatory neuropathy (Milligan et al., 2003). This reflects that spinal proinflammatory cytokines are important in the creation and maintenance of neuropathic pain. In addition, suppression of astrocyte and microglial activation by over expression of the anti-inflammatory cytokine IL-10 was shown to reverse inflammation induced paralysis, to reduce disease associated reduction in sensitivity to touch and suppressed CNS glial activation observed in EAE (Sloane et al., 2009).

To further understand the immune mechanisms associated with differential EAE severity observed between the two treatment groups, T cell responses to MOG₃₅₋₅₅ antigen restimulation were observed. The *in vitro* cytokine profile analysis of lymphocytes showed that JNJ 7777120 treated EAE mice produced significantly more IFN γ ; IFN γ is a product of macrophages and during inflammation, it acts in an autocrine manner as a macrophage activator (Luo and Chen, 2012). Similarly, JNJ 7777120 treatment appeared to have a pro-inflammatory effect by reducing levels of the anti-inflammatory cytokine, IL-4 and IL-10 levels. IL-4 and IL-10 have an anti-inflammatory role within the CNS and this has been demonstrated in the mouse EAE model, where mice that are IL-10 $-/-$ and IL-4 $-/-$ exhibited a more severe clinical disease (Bettelli E, 1998, Ponomarev et al., 2007). IL-4 is suggested to regulate inflammation by influencing the phenotype and function of macrophages (Ponomarev et al., 2007). In support of this, treatment of mice with IL-4 in an adoptive transfer resulted in amelioration of EAE (Racke et al., 1994) whereas administration of IL-10 during the induction phase could suppress the clinical signs of

disease (Rott et al., 1994). IL-10 plays a central role in down-regulating IFN- γ and TNF- α production by auto-reactive T cells (Bettelli E, 1998) and lymphocytes and splenocytes from JNJ 7777120 treated EAE produce less IL-10 but have higher IFN γ levels compared to untreated EAE controls.

It is plausible that the increase in activated spinal cord microglia and astrocytes caused by H₄ receptor blockade, led to an increase in CNS IL-4 levels. Microglia and IL-4 mRNA increase during EAE inflammation in response to signals either from oligodendrocytes undergoing cell death or activated astrocytes (Ponomarev et al., 2007). The outcome would be the increased production of an anti-inflammatory cytokine with the potential to regulate the pro-inflammatory activity of astrocytes. IL-10 is an anti-inflammatory cytokine that suppresses proinflammatory cytokine expression, from transcription to protein release, downregulates proinflammatory cytokine production (Moore et al., 2001). This approach has been shown to block and reverse pain arising from spinal inflammation. IL-6 production is higher in the splenocytes than the lymph nodes and this could be a result of increased macrophage expansion as macrophages are capable of releasing IL-6.

Infiltrating leukocytes are critical for the establishment of spinal sensitisation in EAE and the abundant infiltration of T cells and monocytes has been observed (Aicher et al., 2004b). The *in vivo* analysis of T lymphocytes infiltrating the spinal cord of the EAE mice revealed the presence of H₄ receptor positive cells, CD11c dendritic cells and macrophages. An increase in CD4⁺ H₄R⁺ T cells in JNJ 7777120 treated mice was observed, although not statistically significant. H₄ receptor activation has been shown to stimulate actin polymerisation, upregulation of adhesion molecules on the cell surface of leukocytes, chemotaxis and subsequent inflammation (Hofstra et al., 2003, Ling et al., 2004). Cervical lymph node mononucleocytes show a higher proportion of CD3⁺ T cells in the JNJ 7777120 treated EAE mice, with suppressed expression of MNCs. However these cells are in low populations in the spinal cord which hosts a higher population of H₄R⁺ dendritic cells, H₄R⁺ T cells and macrophages. CD3⁺ T cells accumulate within the dorsal horn of mice with EAE early in the disease course when cold and tactile allodynia are observed (Olechowski et al., 2009) whereas neuropathic tactile allodynia is reduced in T cell deficient *rag1*-null mice.

Immunoreactivity of T cells, astrocytes, macrophages and microglia present in the dorsal horn is significantly up-regulated during EAE suggesting that inflammation and reactive gliosis in the spinal dorsal horn may be the crucial mechanism underlying the behavioural hypersensitivity observed in MOG₃₅₋₅₅ EAE model. Due to the little evidence for direct injury to the peripheral nervous system in MOG₃₅₋₅₅ induced EAE model, the most likely source of the activation of spinal gliosis in the disease is from trafficking of T cells into the CNS (Costigan et al., 2009). T cells have the ability to penetrate the BBB, enter the dorsal horn and are integral for the generation and maintenance of central sensitisation and neuropathic pain. Athymic “nude” mice lacking mature T cells do not develop neuropathic pain after peripheral nerve injury (Moalem et al., 2004). Our results show an abundance of CD3⁺ T cells in the superficial dorsal horn, correlating with the development of allodynia and these most likely affect sensory neurons through the release of pro-inflammatory cytokines that may directly sensitise dorsal horn neurons. T cells can also secrete neurotrophins like brain-derived neurotrophic factor (BDNF) and this may explain the mechanism of immune-mediated neuroprotection following axonal injury (Serpe et al., 2005).

Our results are in accordance of those of Del Rio *et al.* (del Rio et al., 2012) who showed direct evidence of a role for H₄ receptor in the modulation of EAE severity. In this report, H₄ receptor KO mice developed more severe EAE despite having T effector cell responses that were equal to those of wild type mice. The H₄ receptor KO mice presented with augmented neuroinflammation and increased BBB permeability compared with wild type mice, despite comparable T effector cell responses. During disease progression, despite increased H₄ receptor expressing immune cells in the CNS, antagonism of the H₄ receptor would lead to a reduction in the absolute number of T regulatory cells as well as those in the CNS, as was the case with H₄ receptor KO mice.

Immunised H₄ receptor KO mice showed a higher proportion of T helper 17 (Th17) memory cells within the CNS, consistent with fewer regulatory T cells (T_R) due to the presence of a defect in the migration capacity of the lymphocytes. H₄ receptor activation using 4-methylhistamine has been shown to influence T_R migration into

the lung (Morgan et al., 2007). Our data and that of Del Rio *et al.* (2012) further demonstrate that histamine signals through the H₄ receptor to induce T_R migration to the site of inflammation. We cannot exclude the possibility that the increased presence of H₄ receptor signalling in the CNS contributes to disease severity as a result of disrupted CNS-central function. The H₁-and H₃ receptors are also expressed in the CNS and histamine may be mediating its effects on hyperalgesia through these receptors as they remain unaffected by the selective and highly specific H₄ receptor antagonist.

H₄ receptor signalling appears to negatively regulate EAE by controlling the infiltration and suppressive actions of T_R cells during the acute phase of disease. This is critical in regulating the proliferation and expansion autoreactive pathogenic T cells. The observation that H₄ receptor blockade with JNJ 7777120 and H₄ receptor KO results in severe EAE compared to WT mice reflects the importance H₄ receptor-mediated T_R cell migration in controlling the inflammatory response in the CNS. However, the use of both central and peripheral acting H₄R agonists may be useful in treating patients with this syndrome.

Apart from the immunological pathology involved in MS, chronic neuropathic pain is a common but little understood symptom associated with the disease. Neuropathic pain caused by lesions and dysfunction of the CNS is the most prevalent and difficult to treat pain syndrome observed in MS patients. MS pain is thought to represent pain as a result of lesions of CNS regions that process pain, but it could also be due to a loss of descending inhibitory pathways generating enhanced responses to noxious stimuli (Sandyk, 1999).

Recent evidence has shown the functional localisation of the H₄ receptor in the CNS (Strakhova et al., 2009); located on the soma of small and medium diameter sensory neurons, lamina I-II of the lumbar spinal cord (Connelly et al., 2009) and also on microglia (Ferreira et al., 2012). JNJ 7777120 has been shown to exert analgesic effects on animal models of inflammatory and neuropathic pain (Hsieh et al., 2010a). Therefore, the possibility that antagonism of central H₄ receptor contributes to the exacerbation of EAE cannot be excluded.

Several studies have showed that expression of the neuropeptides CGRP and galanin, which are located in the dorsal horn of the spinal cord, is elevated (via increased receptors or neuron sprouting) during neuropathic pain (Bennett et al., 2000) caused by spinal cord injury and peripheral nerve damage and inflammation. Galanin expression in the dorsal horn is elevated during neuropathic pain caused by peripheral nerve injury (Liu and Hokfelt, 2002b). However, neither neuropeptide is elevated at any time point during MOG₃₅₋₅₅ model of EAE (Olechowski et al., 2009) therefore changes in sensory processing during disease progression cannot be accounted for by different expression patterns of these neuropeptides.

CGRP mRNA levels increase in the motor neurons during the acute phase of EAE (Giardino et al., 2004) and this accumulation is attributed to increased synthesis, although accumulation because of the blocking of peripheral transport cannot be excluded (Caldero et al., 1992). Our analysis of CGRP expression on putative A δ fibres expression showed [apparent] up-regulation of CGRP staining in the superficial dorsal horn of JNJ 7777120 treated EAE. Previous studies have shown that substance P-mediated signalling contributes to the maintenance of inflammation in the CNS during the chronic phase of EAE and EAE severity is reduced in NK1-/- animals (Reinke et al., 2006).

Although JNJ777120 has been proven to have functional antagonism on calcium mobilisation and chemotaxis in leukocytes through the inhibition of adenylyl cyclase activation and cAMP (Thurmond et al., 2004b), it may have agonist properties in the CNS through activation of a pathway that does not involve calcium mobilisation. Rosethorne and Charlton (2011) have shown that JNJ777120 is a biased agonist at the human H₄ receptor, acting as a neutral antagonist for [³⁵S] GTP γ S binding and a partial agonist for β -arrestin recruitment. It is possible that in the EAE model, JNJ 7777120 activates central H₄ receptors through this mechanism. This is attributed to the existence of multiple active conformations, and each compound's ability to stabilize a subtly different state over another and therefore activating a different pathway. They demonstrated that although JNJ 7777120 is an antagonist regarding G protein-dependent signalling, it acts as an agonist in a non-G protein-dependent

manner to recruit β -arrestin to the receptor, as demonstrated by the lack of sensitivity to pertussis toxin treatment (Rosethorne and Charlton, 2011).

Due to the expression of the H_4 receptor in the CNS, we cannot exclude the possibility that the absence of H_4 receptor signalling contributes to the increased disease severity, because of disturbed CNS signalling functions. Our current findings show that H_4 receptor antagonism negatively regulates EAE by enhancing the infiltration of leukocytes and possibly, the suppressive activity of T_R cells within the CNS. We hypothesise that increased CNS inflammation as a result of H_4 receptor antagonism enhanced hyperalgesia and allodynia in this EAE model, because reduced inflammation reverses disease induced hyperalgesia and allodynia (Sloane et al., 2009). Similarly, H_4 receptor KO mice develop more severe EAE than WT mice, highlighting the importance of temporal localisation of T_R cells. On the other hand, JNJ 777120 could be acting as an agonist via the recruitment of β -arrestin, therefore worsening the clinical state of the animals. It is possible that the agonism and antagonism of the centrally expressed H_4 receptor may have different effects compared to the peripherally expressed H_4 receptor. Further work is needed for elucidating the pathway activated during central H_4 receptor agonism and antagonism, if specific H_4 receptor ligands are to be among the group of potential drugs to treat inflammatory or autoimmune diseases and neuropathic pain secondary to CNS inflammatory disorders.

The next chapter will investigate the central effects of H_4R antagonism using a series of state-of-the art novel behavioural tests. This will validate the use or otherwise of centrally active H_4R targeted analgesic drugs.

Chapter 6

The behavioural effects of histamine H_4 receptor antagonism

6.1 Objectives

The objective was to determine the potential central role of the histamine H₄R in a range of behaviours, including anxiety, learning and memory. One of the aims was to determine if there were any changes in anxiety behaviours in CD1 (lo anxiety strain) and Balb/c (high anxiety strain) mice in the elevated platform test developed in our laboratory, following administration of the selective H₄R antagonist JNJ 7777120. Another aim was to investigate the effects of H₄R antagonism in motor activity, learning and memory in Balb/c mice.

6.2 Introduction

Strakhova *et al.* (2009) showed that in humans, H₄R expression is highest in the spinal cord, followed by hippocampus, cerebellum and other brain regions (i.e. cerebellum, hypothalamus, amygdale, thalamus, and cortex). The Immunohistochemical detection of H₄ receptors but lack of mRNA in the cortex implies that H₄ receptors are expressed on fibres emanating from other brain regions. Similarly, H₄ mRNA is expressed in the rat cortex, cerebellum and brain stem. Further immunohistological studies showed mouse H₄ expression is evident in lamina I, II, III, IV and V of the cortex (peak in lamina IV) and the hippocampal formation (Connelly et al., 2009). This expression was shown to be functionally active through activation within layer IV neurons with 4-Methylhistamine; this effect was blocked by the selective H₄ receptor antagonist JNJ10191584 (Connelly et al., 2009). This pattern of expression is similar with that of the H₃R such as, overlap in the entorhinal cortex and deep lamina of the cortex but contrasts particularly with the high H₄ expression in the thalamus.

Due to the distribution of H₄ receptors in critical areas of the brain, it is worthwhile to examine its possible involvement in behaviour.

In the present study, we examined the effects of a H₄ antagonist on a range of behaviours in mice. We used the elevated platform with slopes developed by our laboratory (Ennaceur *et al.*, 2010) and the novel object recognition (NOR) task

developed by Ennaceur and Delacour (Ennaceur and Delacour, 1988). The first test consists of an elevated platform with steep slopes attached on two opposite sides. It has been demonstrated in this test that C57BL/6J and CD-1 mice do cross onto the slopes while Balb/c mice do not cross, but spend most of the test duration in the area adjacent to the slopes. This indicates that Balb/c mice display high levels of anxiety compared to C57BL/6J and CD-1 mice. Diazepam (clinically used anxiolytic) induced, in dose-dependent fashion, the Balb/C mice to enter the slopes indicating its anxiolytic effects (Michalikova et al., 2010). The NOR test is performed in an open-field and performed in two stages. In the initial sample phase, animals are exposed to two identical objects. In the second stage, choice phase, animals are exposed again to two objects, one familiar, a copy of the one seen before, and the other novel, never seen before. A delay interval is interposed between the two test phases. At short (up to 1hr) but not at longer retention interval most animals discriminate between the two objects, they spend more time engaged with the novel than the familiar object. This latter test has been translated successfully to human clinical trials.

6.3 Experiment 1

6.3.1 Materials and Methods

6.3.1.1 Animals: 32 BALB/c and 32 CD-1 male mice obtained from Charles River (UK) were used in the experiments described in this report. They were 56-62 days old at the date of arrival and were left to acclimatize to local laboratory conditions for one week. They were housed in a colony room that was held under a 12 h light/12 h dark cycle (light 0700-1900 h at 40 Lux) and at 23°C \pm 1. In order to avoid unequal light exposure, the upper shelf was occupied with plastic cages filled with clean sawdust. Mice were housed in a group of 4 per cage. Individual mice could be identified by their cage number and their ear tag code. All mice had *ad libitum* access to food and water. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK.

6.3.1.2 Drugs and treatments:

JNJ 7777120 was purchased from Tocris (UK). It was dissolved in PBS with 2.5% dimethyl sulphoxide (DMSO). Both JNJ 7777120 and saline were freshly prepared on the days of the test and administered intraperitoneally (i.p) 30 minutes before the start of the test session in a volume of 10 ml/kg. Control mice received PBS with DMSO. The experiment consisted of 4 groups from each strain of mice. In each strain, one group was treated with saline (n=8), and separate groups were treated with JNJ777120 at 5 mg/kg (n=8), 10 mg/kg (n=8) and 20 mg/kg (n=8).

6.3.1.3 Apparatus and testing procedures:

It consists of a platform 80 cm x 80 cm wide, elevated 75 cm from the ground. Steep inclined panels made of rigid wire mesh are attached on two opposite sides of the platform (width: 80 cm x 25 cm). The platform was divided into a central area covered with a white tile (16 x 16 cm wide and 0.4 cm thick), an inner area surrounding the central area (16 cm wide), and an outer area (16 cm wide). The outer area was further divided into areas adjacent to the slopes and areas adjacent to void space. The surface of the platform was cleaned to minimize the effects of lingering olfactory cues. The illumination on the surface of the elevated platform was 40 Lux. Mice were transported in a small bucket to the test apparatus where they

were tilted gently onto the tile, which forms the central area of the platform. They were left to explore the apparatus for 12 minutes.

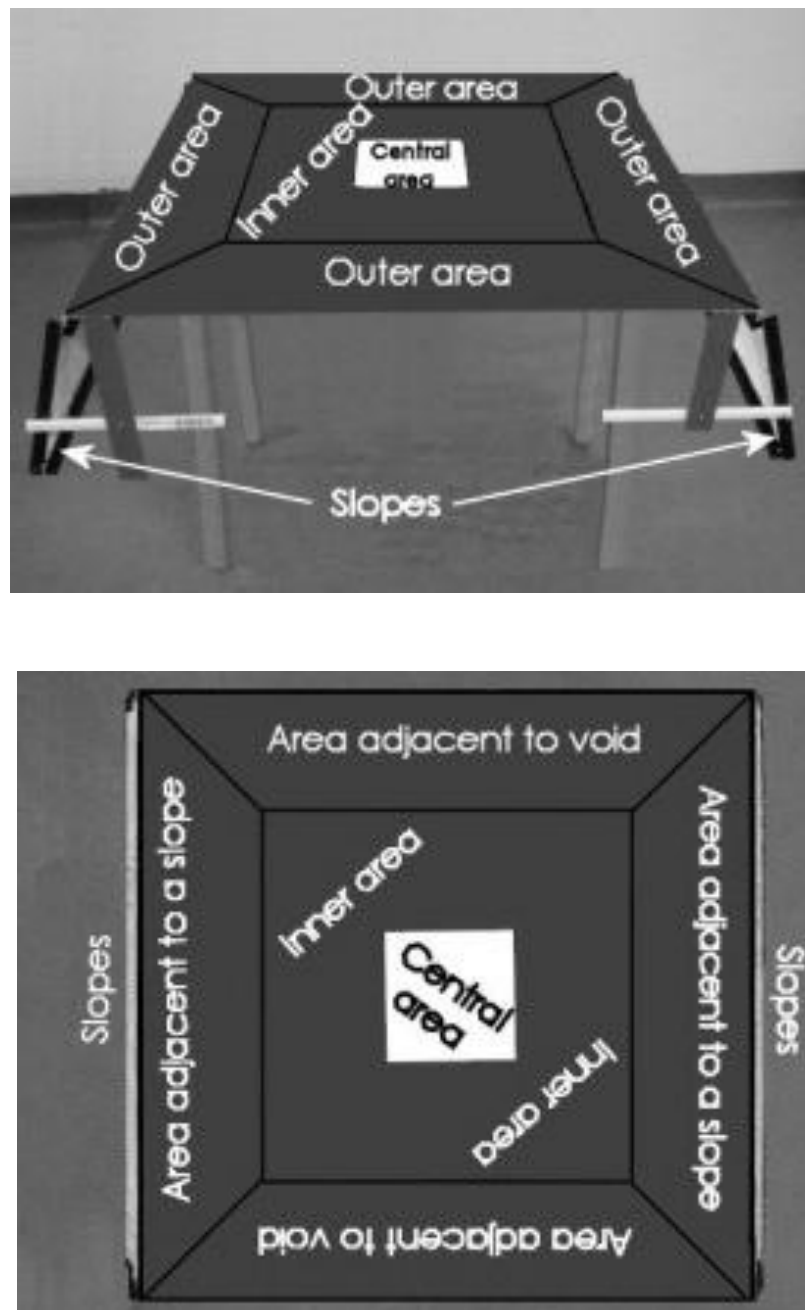


Figure 6.1 Description of the different areas of the elevated platform (top and frontal views)

The recorded data consists of measures of the number of entries and duration into the central area, the inner area, the outer area and onto the slopes in addition to latency of first entry into these areas.

The record of outer area was further divided into measures related to areas adjacent to the slopes and areas adjacent to the void space

6.3.1.4 Measurements and data analysis:

The test session was video recorded and the behaviour of rats was analyzed with an in-house computer program, EventLog. The recording of the behaviour of rats was based on entries into defined areas of the apparatus. An entry was recorded whenever a rat crossed with all four paws into an area. EventLog records in sequential order the start and end of each crossing into an area of the open-field. It provides measures of latency, frequency and duration of entries.

All data are expressed as mean \pm S.E.M. Differences among group means values for each measurement were tested for significance with one-way ANOVA followed up with Newman–Keuls post-hoc comparisons (Statistica for Windows, version 5.5). Results were considered significant when $p \leq 0.05$.

6.3.2 RESULTS:

6.3.2.1 Number of crossings on the platform: There were significant differences between groups ($F_{7,56}=20.08$, $p<0.0001$). Saline and drug-treated Balb/c made significantly few crossings compared to saline and JNJ-treated CD-1 mice ($p<0.01$). Balb/c treated with JNJ 10 and 20 mg made significantly few crossings compared to Balb/c saline and Balb/c treated with JNJ 5 mg ($p<0.03$).

6.3.2.2 Crossings onto central and inner areas:

There were significant differences between groups for the number of crossings onto the central ($F_{7,56}=3.35$, $p<0.005$) and inner ($F_{7,56}=5.13$, $p<0.0001$). Balb/c treated with JNJ 7777120 10 and 20 mg made significantly less crossings onto the inner and central areas compared to Balb/c treated with saline and JNJ 7777120 5 mg, and to both saline and drug treated CD-1 mice ($p<0.05$).

The latency of first crossing onto the inner area was not significant between groups ($F_{7,56}=2.03$, $p<0.07$). There were however, significant differences between groups in the time spent in the central area ($F_{7,56}=6.02$, $p<0.009$), inner area ($F_{7,56}=12.32$, $p<0.0001$). Balb/c mice treated with saline and Balb/c treated with JNJ 7777120 5 mg spent more time in the inner and central areas compared to all other groups ($p<0.01$).

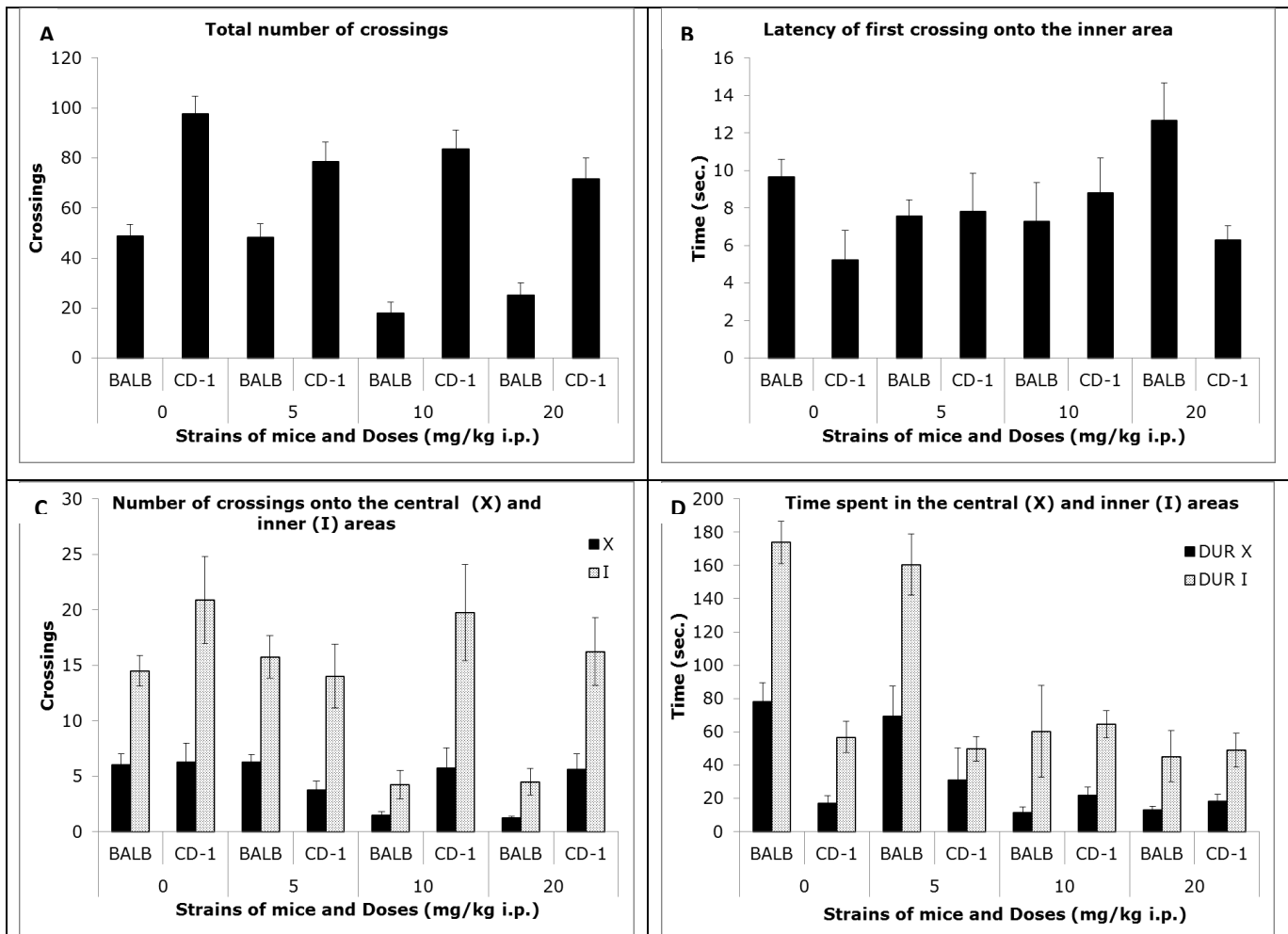


Figure 1a: Saline and JNJ treated Balb/c mice compared to saline and JNJ treated CD-1 mice ($p < 0.01$). Balb/c treated with JNJ 10 and 20 reduced total number of crossings compared to Balb/c treated with saline or treated JNJ 5 mg ($p < 0.03$); **Figure 1b:** No significant differences between groups ($p < 0.07$); **Figure 1c:** Balb/c treated with JNJ 10 and 20 mg compared to Balb/c treated with saline and JNJ 5 mg, and to both saline and drug treated CD-1 mice ($p < 0.05$) made less crossings in the inner and central areas; **Figure 1d:** Balb/c mice treated with saline and JNJ 5 mg spent more time in the inner and central areas compared to all other groups ($p < 0.01$).

6.3.2.3 Number of crossings onto the areas adjacent to void and slopes: There were significant differences between groups for the number of crossings onto the areas adjacent to the void ($F_{7,56}=15.66$, $p<0.0001$) and areas adjacent to slopes ($F_{7,56}=22.37$, $p<0.0001$). Saline and JNJ 7777120 treated Balb/c mice made significantly few crossings onto the areas adjacent to the void and the slopes compared to saline and JNJ 7777120 treated CD-1 mice ($p<0.01$). CD-1 treated with saline made also significantly few crossings compared to CD-1 mice treated JNJ 20 mg ($p<0.01$). Balb/c treated with JNJ 7777120 10 mg made also significantly less crossings on both areas compared to Balb/c treated with saline or JNJ 7777120 5 mg ($p<0.05$).

There were significant differences between groups for the time spent in the areas adjacent to void ($F_{7,56}=2.13$, $p<0.05$) and areas adjacent to slopes ($F_{7,56}=12.20$, $p<0.0001$). The time spent in the areas adjacent to the void space was low but not significant in Balb/c mice treated with JNJ 7777120 10 compared to Balb/c treated with saline ($p<0.09$), JNJ 7777120 5 ($p<0.09$) and JNJ 20 ($p<0.06$). It was also high but only significant compared to CD-1 mice treated with JNJ 7777120 5 and 20 mg ($p<0.05$). The time spent in the areas adjacent to the slopes was significantly high in Balb/c treated with JNJ 7777120 10 and 20 compared all other groups ($p<0.001$).

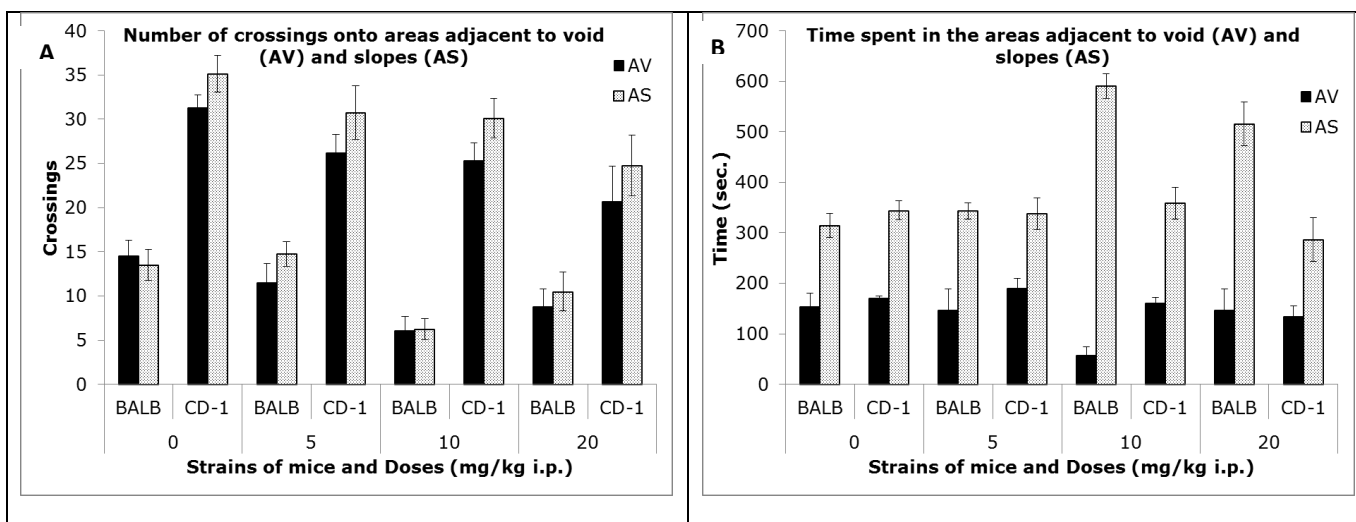
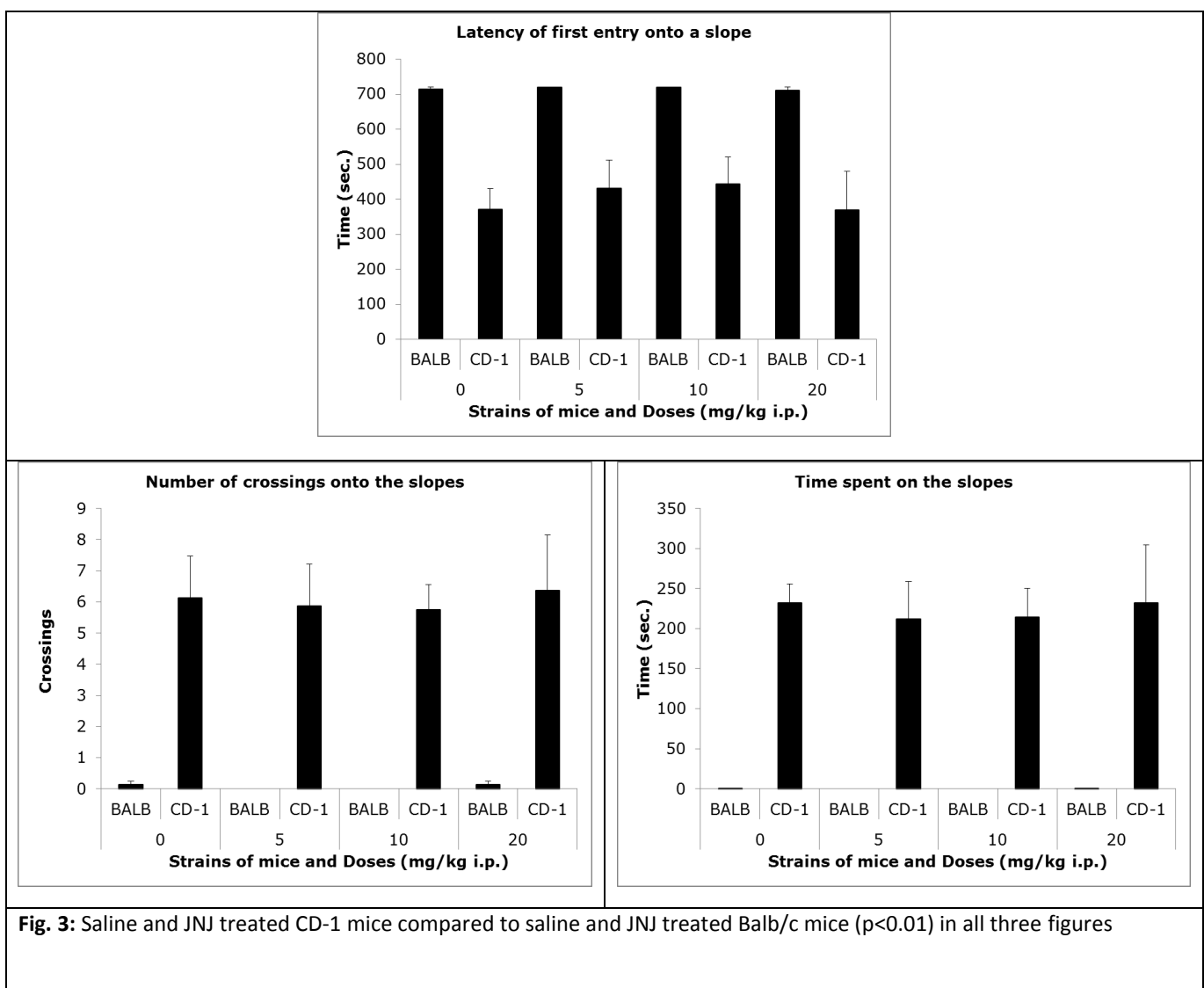


Figure 2a: For the areas adjacent to void and slopes - Saline and JNJ treated Balb/c mice made few crossings compared to saline and JNJ treated CD-1 mice ($p < 0.01$). CD-1 treated with saline made few crossings compared to CD-1 mice treated JNJ 20 mg ($p < 0.01$). Balb/c treated with JNJ 10 mg made the fewest number of crossings compared to Balb/c treated with saline or JNJ 5 mg ($p < 0.05$).

Figure 2b: For the areas adjacent to void – Balb/c mice treated with JNJ10 was low but not significant compared to Balb/c treated with saline ($p < 0.09$), JNJ 5 ($p < 0.09$) and JNJ 20 ($p < 0.06$), and compared to CD-1 mice treated with JNJ 5 and 20 mg ($p < 0.05$).

For the areas adjacent to slopes – Balb/c treated with JNJ 10 and 20 compared all other groups ($p < 0.001$).

6.3.2.4 Crossings onto the slopes: There were significant differences between groups in the number of crossings onto (F7,56=4.95, $p<0.0002$) and time spent on the slopes (F7,56=6.56, $p<0.0001$). There were also significant differences between groups in the latency of first crossing onto a slope (F7,56=6.76, $p<0.0001$). In fact, all saline and drug-treated CD-1 mice crossed onto the slopes whereas only two Balb/c mice did cross onto the slopes. One Balb/c was from the saline group and the other was from JNJ 7777120 20mg. The duration of visits for these two mice was less than 4sec.



6.4 Experiment 2

6.4.1 MATERIALS AND METHODS:

6.4.1.1 Animals: 32 BALB/c male mice obtained from Charles River (UK) were used in the experiments described in this report. They were 56-62 days old at the date of arrival. All conditions as described in experiment 1.

6.4.1.2 Drugs and treatments:

JNJ 7777120 was purchased from Tocris (UK). It was dissolved in PBS with 2.5% dimethyl sulfoxide DMSO. Both JNJ 7777120 and saline were freshly prepared on the days of the test and administered i.p. 30 minutes before the start of the test session in a volume of 10ml/kg. Control mice received PBS with DMSO. The experiment consisted of 4 groups: saline (n=8), and JNJ 777120 at 5 mg/kg (n=8), 10 mg/kg (n=8) and 20 mg/kg (n=8).

6.4.1.3 Apparatus and testing procedures: It consisted of an open-field 80 cm x 80 cm wide and 50 cm height. The floor was divided into nine equal squares. The central square was covered with a white tile. It was cleaned to minimize the effects of lingering olfactory cues. The illumination on the floor was 40 Lux. Mice were transported in a small bucket to the test apparatus where they were tilted gently onto the tile.

Mice were first habituated to the open-field in 3 consecutive sessions, one session a day. Each session lasted 12 minutes. In the fourth day, mice were exposed to two identical objects, each located at one of the back corner of the box apparatus. The session lasted 10 minutes. In the fifth day, mice were re-exposed to two objects in the previous corner locations. One of the objects was an identical copy of the one shown in the previous session and the other was completely novel. The location of the familiar and novel object was counterbalanced between mice.

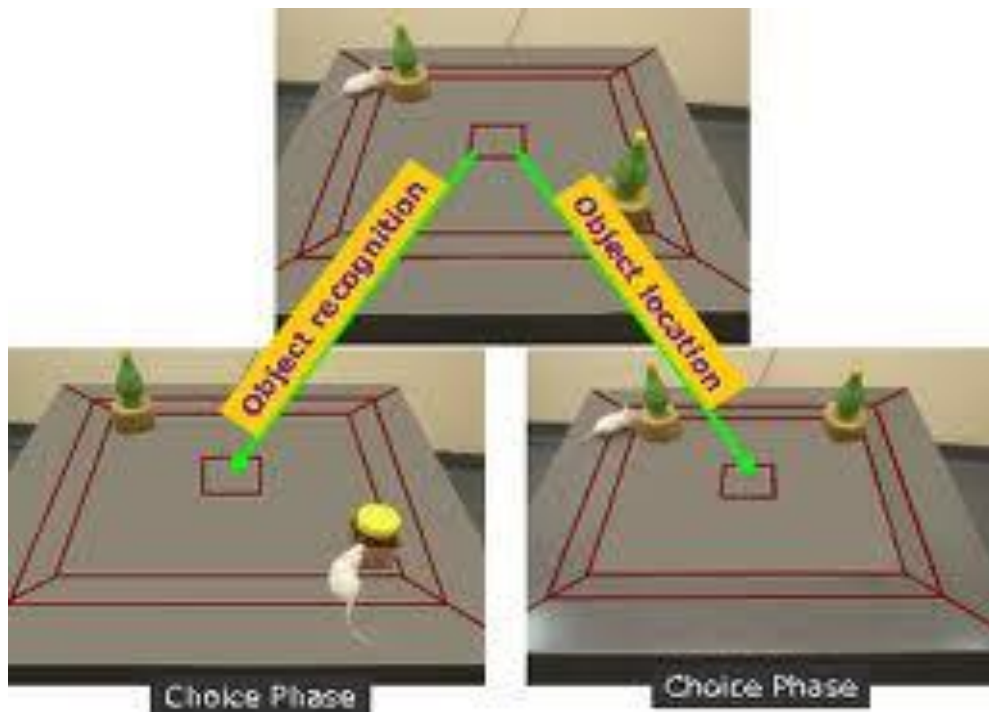


Figure 6.2 The layout for the object recognition experiment.

During the sample phase (top panel), the mouse is expected to spend more time closer to the object than it does in any other areas of the elevated platform. During the choice phase (bottom panels), a novel object is introduced to the elevated platform in addition to the original familiar object. Object recognition is observed when the mouse spends less time with the familiar object (because it remembers the object from memory) and more time with the novel object.

6.4.1.4. Measurements and data analysis: see experiment 1,

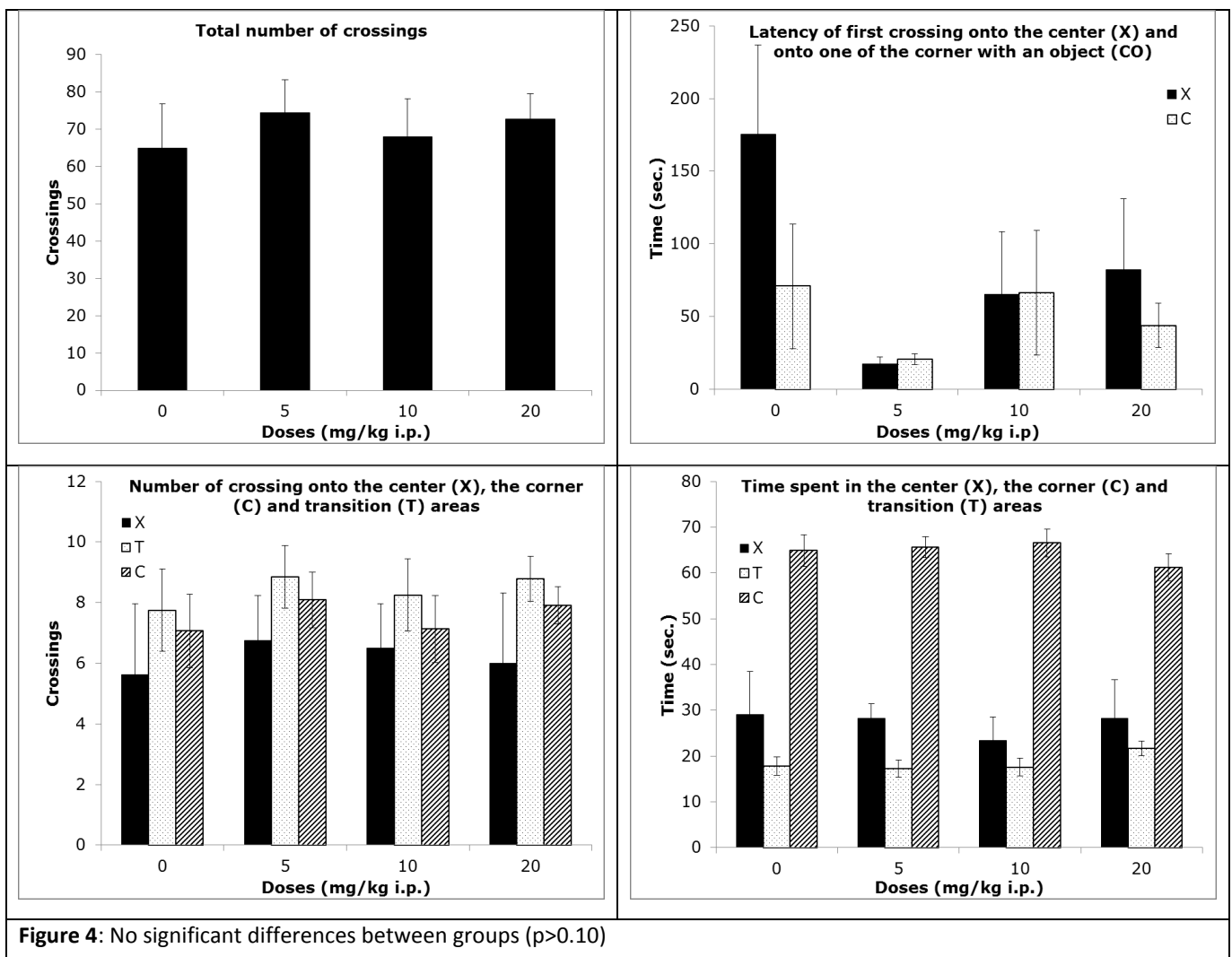
All data are expressed as \pm S.E.M. Differences among group means values for each measurement were tested for significance with a one-way ANOVA followed up with Newman–Keuls post-hoc comparisons (Statistica for Windows, version 5.5). Discrimination between novel and familiar objects was assessed using paired student t-test for each group. Results were considered significant when $p \leq 0.05$.

6.5 OBJECT RECOGNITION

6.5.1 Sample phase

During the sample phase, animals are exposed to two identical objects.

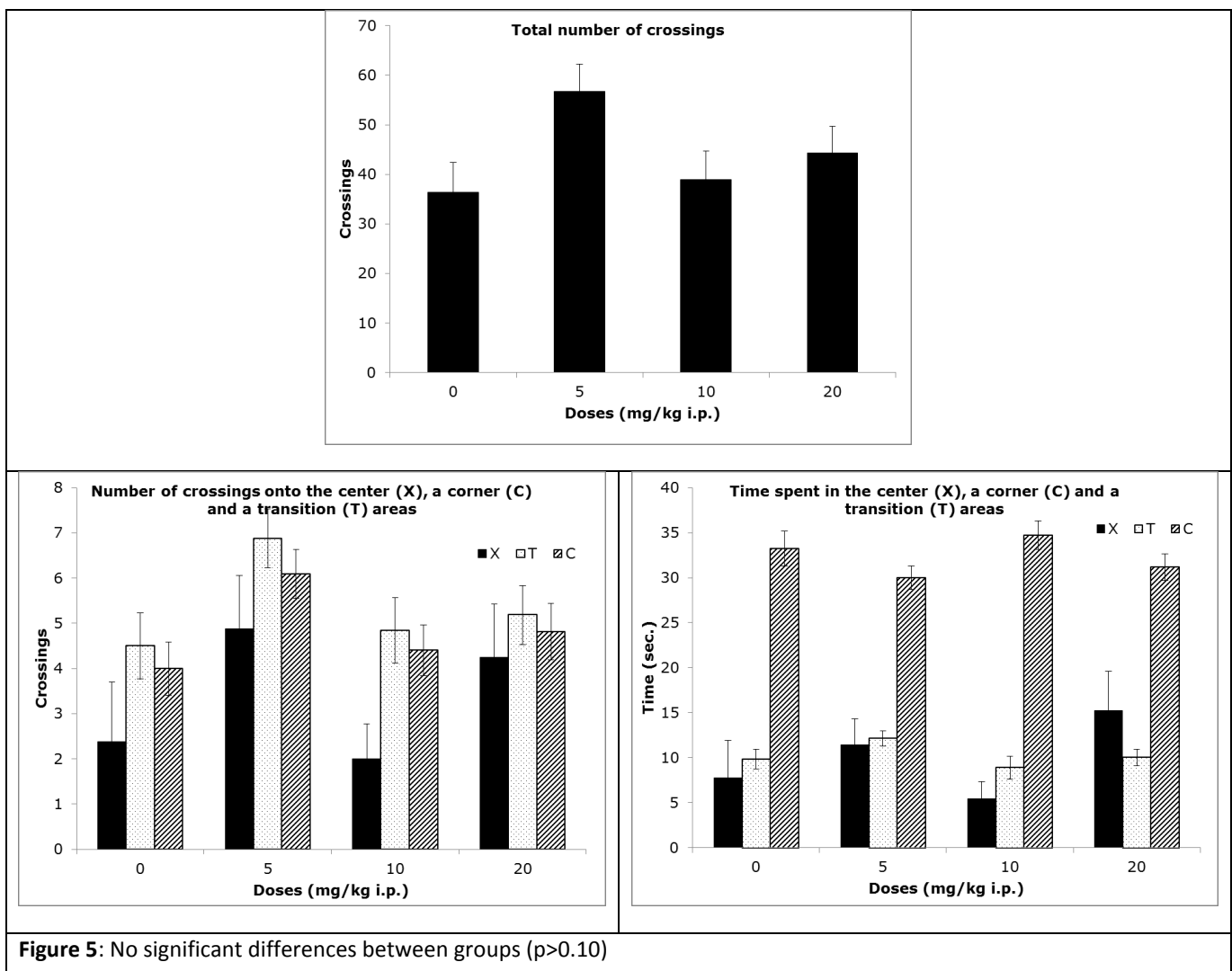
There were no significant differences between groups on the total number of crossings ($F_{3,28}=0.21$, $p>0.10$), on the number of crossings onto the center ($F_{3,28}=0.07$, $p>0.10$), corner ($F_{3,28}=0.21$, $p>0.10$) and transition ($F_{3,28}=0.29$, $p>0.10$) areas. There were also no significant differences between groups in the time spent in the center ($F_{3,28}=0.14$, $p>0.10$), corner ($F_{3,28}=0.61$, $p>0.10$) and transition ($F_{3,28}=1.26$, $p>0.10$) areas.



6.5.2 Choice phase

During the choice phase, animals are exposed again to two objects, one familiar, a copy of the one seen before, and the other novel, never seen before.

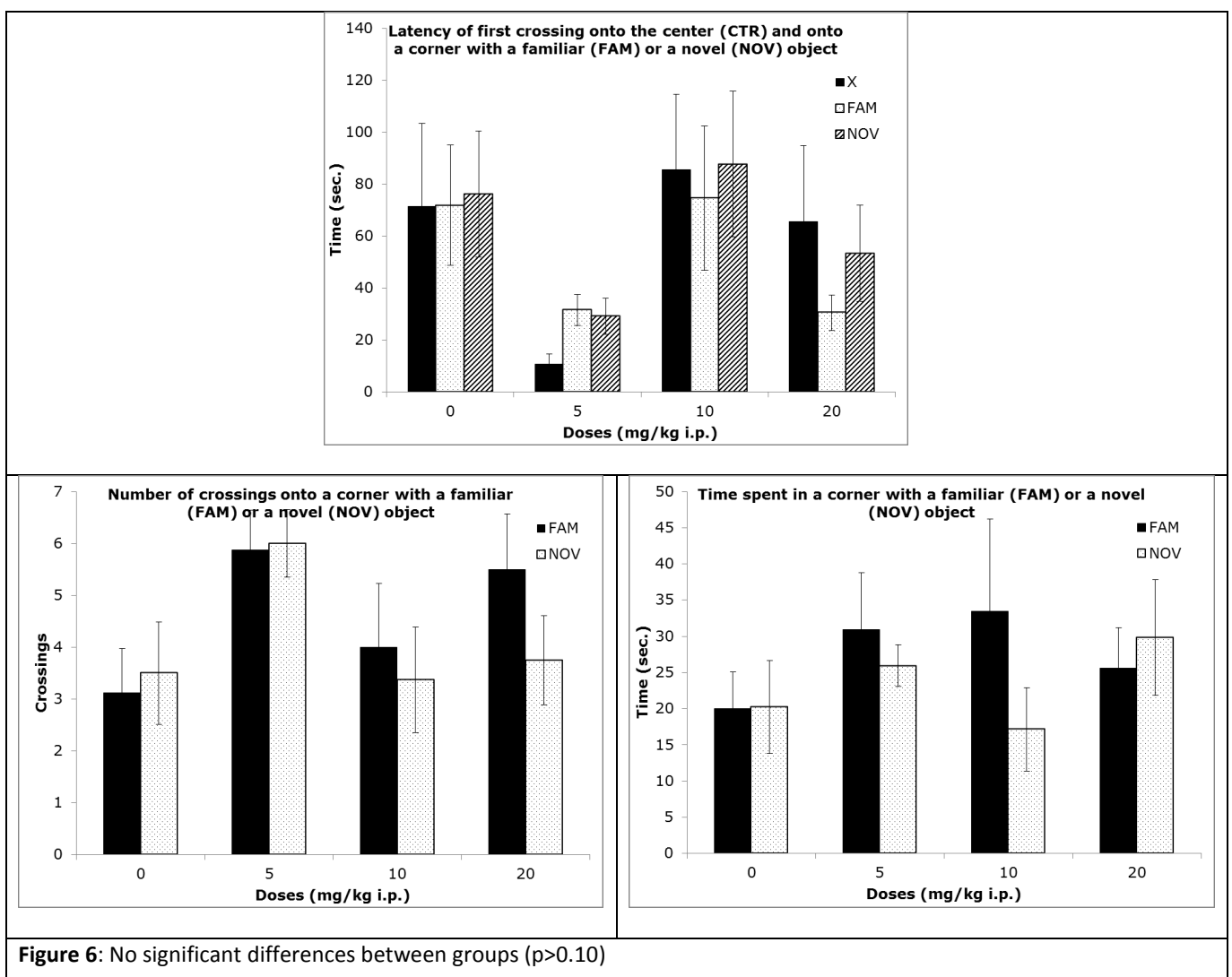
There were no significant differences between groups on the total number of crossings ($F_{3,28}=2.57$, $p>0.07$), on the number of crossings onto the centre ($F_{3,28}=1.52$, $p>0.10$), corner ($F_{3,28}=2.44$, $p>0.08$) and transition ($F_{3,28}=2.33$, $p>0.09$) areas. There were also no significant differences between groups in the time spent in the centre ($F_{3,28}=1.51$, $p>0.10$), corner ($F_{3,28}=1.74$, $p>0.10$) and transition ($F_{3,28}=1.76$, $p>0.10$) areas.



6.5.3 Discrimination

There were no significant differences between groups in the latency of first crossing onto the center ($F_{3,28}=1.59$, $p>0.10$) and onto the corners with a familiar ($F_{3,28}=1.71$, $p>0.10$) or novel object ($F_{3,28}=1.52$, $p>0.10$).

There were also no significant differences between groups in the number of crossings onto and time spent in the corner with a familiar ($F_{3,28}=0.53$, $p>0.10$ and $F_{3,28}=0.51$, $p>0.10$, respectively) and the corner with a novel ($F_{3,28}=0.43$, $p>0.10$ and $F_{3,28}=0.88$, $p>0.10$, respectively) object.



The table below indicates that neither mice treated with saline nor mice treated with JNJ discriminated between objects.

		Crossings		Time	
Doses mg/kg		t	p	t	p
0	n=8	0.6	>0.10	0.03	>0.10
5	n=8	0.17	>0.10	0.52	>0.10
10	n=8	0.96	>0.10	1.73	>0.10
20	n=8	0.97	<0.02	0.48	>0.10
Table 1: p values for the number of crossings (see fig. 6b) onto and time spent ((see fig. 6c) in the corners occupied with a familiar object compared to corners occupied with a novel object.					

6.6 Discussion

The role of the histamine H₄R in the brain is poorly understood, despite its distribution in critical areas of the brain. Therefore, it is important to examine its potential role in motor activity, anxiety and memory. This chapter describes the behaviour of mice in an anxiety test based upon exposure to an elevated platform with steep panels attached on two opposite sides. Anxiety is defined as the result of a conflict between the drive to approach a threatening stimulus and the drive to avoid the same stimulus.

The elevated platform is divided into the central area covered with a thin white tile, an inner area surrounding the central area and an outer area. The outer area is further divided into areas that are adjacent to the downward slopes and areas adjacent to the void space. The surface of the inner area is equal in size to that of the outer area, validating the comparisons between these two areas. The central area defined by the white tile was justified by reports that the open space of an open-field is anxiogenic to the animals, preventing them entering the space. Minus a tile, the central area has no physical boundaries and the tile is sufficient to attract both rats and mice to explore the central area. During the test, anxious animals were expected to make few or no entries onto the downward slopes, and to spend most of their time away from the platform edge.

Balb/c mice treated with JNJ 7777120 at 10 mg and 20 mg made fewer crossings onto the inner and central areas and spent less time in these areas, compared to the CD-1 mice. In the elevated platform, JNJ 7777120 at 10 and 20 mg appears to affect the behaviour of Balb/c mice but there is no indication of reducing anxiety. Their total number of crossings on the platform was very low. This behaviour was observed in a previous study (Ennaceur et al., 2010b). These results show that JNJ 7777120 administration did not facilitate crossing onto the slopes in Balb/c mice, as only one animal from the control and treated groups crossed onto the slopes. Similarly, it did not affect the crossings of CD-1 mice.

Our results show that the saline and JNJ 777120 animals made fewer entries and spent less time in the inner and central areas of the platform. This contrasts with previously published data which demonstrated that both Balb/c and CD-1 mice made frequent entries, and spent more time in the outer and inner areas of the platform (Michalikova et al., 2010). Frequent entries to these areas represent a response strategy adopted by animals in an unfamiliar environment, as they look for an escape route and assess their safety. In an open space lacking a protective area to escape to, anxiety is experienced in any part of the apparatus and the slopes provide an escape route, but are also a handicap that the animals need to overcome. In this model, the behaviour of the animals is controlled by the natural desire to explore the different areas of the platform and slopes, as well as the drive to escape from the platform and the slopes. Therefore, if they want to find an escape route, they need to explore. Despite the 12 minute length of a session, animals remained active and did not show signs of decreased motor exploration when exposed for three sessions or more to the apparatus.

This test is comparable to the open-field, the plus-maze and the light dark box which measure fear-induced avoidance (Dere et al., 2002). It has been suggested that different areas of the elevated platform with downward slopes induce different degrees of anxiety, especially when animals move away from a protected space. In the elevated platform, the slopes provide an incentive for the animals as they can cross onto the platforms and get closer to the ground. Mice exposed to an elevated platform try to escape by jumping onto the ground when the platform is not sufficiently raised (unpublished data). Therefore, the platform is raised by 75 cm from the ground and animals show preference for the areas adjacent to slopes which provide a potential route for escape. Balb/c mice appear to be more anxious than CD-1 and c57 mice as they did not cross onto the slopes. This cannot be accounted for by physical abilities because a previous study using diazepam treated Balb/c mice did cross frequently and spent more time on the slopes (Ennaceur et al., 2010a). The least anxious strains of mice, the CD-1 cross onto the slopes and venture to the far end of the slopes to get closer to the ground as do diazepam treated Balb/c mice. Our results show that unlike the saline and drug treated CD-1 mice which crossed

onto the slopes, Balb/c mice did not. They spent a significant amount of time in the areas adjacent to the slopes than in areas adjacent to the void, which indicates that the Balb/c mice were aware of the presence of the slopes.

Novel object recognition has been used to assess anxiety and memory in animals. It has previously postulated that when you expose a familiar object alongside a novel object, both young and adult mice approach and spend more time exploring the novel, rather than the familiar object. This is considered an indication that a representation of the familiar object exists in memory. The habituation sessions are necessary because anxiety is predominant during the initial stages and this prevents object recognition, but this subsides following repeated exposures (Ennaceur et al., 2009) thereby allowing the animal to recognize a novel from a familiar object.

In the object recognition test following the habituation, JNJ 7777120 had no effect on any test parameter. It had no effect on the number of crossings and time spent in the centre, corners and transition areas of the platform at the administered concentrations during the sample and choice phase. JNJ 7777120 did not facilitate discrimination between novel and familiar object. In this study, we used a 24 hour delay interval but mice were exposed to the sample object for 10 min. Data from our laboratory using this model demonstrated that rats ventured into the centre of the field, made more approaches to and spent more time on a novel object (Ennaceur et al., 2009). On the other hand, a study in which Balb/c H₄R knockout mice were subjected to spatial learning and memory test in the Morris water maze and showed better performance, compared to the poor performance of the wild type in this experimental setting. The H₄R knockout mice appeared to react with hyperactivity to handling, showing the potential differences in behavioral phenotype between H₄R knockout and wild type mice (Rossbach et al., 2012).

A recent study using the H₄R agonist VUF 8430 reported this drug to dose-dependently prolong the time spent in the light chamber of the light/dark box, indicating its potential anxiolytic effect (Galeotti et al., 2013). On the other hand, the H₄ antagonist JNJ 10191584 had no effect but pre-treatment antagonized the effect of VUF 8430 further confirming selectivity of H₄R activation. However, the H₄R agonist at the dose that increased the time spent in the light compartment reduced

significantly the crossings between the two compartments of light/dark box. In this experiment mice were released in the middle of the light compartment. Hence, it is possible that the time spent in this compartment is due to freezing a response than reduced anxiety. The effects of H₄R activation on memory in the passive avoidance paradigm, using mice with scopolamine impaired memory were also investigated. In the passive avoidance task mice treated with the H₄R agonist at the dose that increased the time spent in the light compartment did not facilitate crossing onto the dark compartment. The authors interpret this as a lack of effect of the drug on memory but with holding a response toward the dark compartment can be considered as indicative of increased anxiety (McNaughton and Corr, 2004).

At present, the role of the central H₄R in anxiety, learning and memory remains unclear. Our results show that antagonism of the H₄R with JNJ 7777120 had no anxiolytic or sedative effects on the Balb/c mice, **hence reduced entry to the center of the platform with increasing JNJ 7777120, cannot be accounted for by reduced motor activity**. However, the work of Galeotti *et al.* (2013) shows the potential anxiolytic effects of receptor activation with the agonist VUF8430. The role of the receptor in memory requires further investigation because JNJ 7777120 administration did not produce any improvements in the novel object recognition memory task, after a 24 hour delay, whereas H₄R knockout mice showed a better performance in the Morris water maze test, compared to the wild type counterparts. There was some doubt on the suitability of the wild-type mice used in the latter study which requires clarity (Johnson & Johnson, personal communication). In order to improve consistency, the same methodology needs to be employed in order to understand the effects of receptor absence, antagonism and activation on anxiety, learning and memory. In conclusion, our studies provides evidence for central H₄Rs (significant effects on entries to central region of elevated platform), but limited evidence for roles in overall motor activity, anxiety or NOR memory.

CHAPTER 7

GENERAL DISCUSSION

During the course of this PhD, the previously generated antibody for the full length histamine H₄ receptor (Shenton, 2007) has been used to locate the expression and distribution of the histamine H₄ receptor in the rodent periphery and central nervous system. H₄R ligands have been used to determine the effects of receptor activation and antagonism. A number of key hypotheses have been addressed:

1) The histamine H₄ receptor is expressed on C-fibre sensory neurones

The H₄ receptor is expressed in the rodent and human CNS with mRNA expression highest in the spinal cord. Immunohistochemical studies confirmed protein expression in the rodent and human spinal cord, mostly observed in lamina I and II of the dorsal horn. Using immunological probes, the H₄R has been detected on substance P-positive C-fibres and CGRP-positive A δ fibres innervating the dermis as well as surrounding the blood vessels in the dermis of the skin. The observation that the histamine-induced itch and the inhibitory effects of the H₄R antagonists were unaltered in mast cell-deficient mice (which have few or no mast cells in skin), indicated that the H₄R on mast cells was not required for initiation of H₄R-mediated pruritus. The observation of the H₄R expression on substance P and CGRP positive neurons in the rat skin supports the hypothesis of a peripheral acting site for the H₄R antagonists which have analgesic (Hsieh et al., 2010a), and anti-pruritic (Dunford et al., 2007).

The double labelling immunofluorescence approach was used to determine the possible co-localisation of H₄Rs on peptidergic C-(substance P) and A δ (CGRP) nociceptive fibres at the level of the skin, dorsal root ganglia (DRG) and the dorsal horn neurons of the spinal cord in the rat. At all levels of the sensory pathway tested, our results show H₄R expression on both C and A δ fibres, with the latter particularly prominent at the level of the skin and DRG. **This expression profile contrasts with the H₃R, which was found to be expressed only on a subset of A δ fibres, but is undetectable on C-fibres** (Cannon, Chazot et al., 2007). Through preliminary functional analysis, we have demonstrated

that the H₄R is functionally expressed on primary rat DRG sensory neurones using the new potent highly selective agonist VUF 8430. This compound is a full agonist at human and rodent H₄R in both endogenous and recombinant H₄R expressing systems (Lim et al., 2009) and selective activation of the H₄R on primary isolated neurons elicits a dose-dependent [Ca²⁺] influx, **providing a robust new useful functional model for studying sensory neuronal H₄ receptors**. The presence of the H₄R on peripheral and central sensory neurones suggests both peripheral and central acting sites for H₄R antagonists that have antinociceptive and antipruritic effects.

2) The histamine H₄ receptor expression is differentially effected in acute and chronic pain conditions

Immunoblotting using the anti-H₄R antibody directed toward the full length receptor was used to determine the changes in H₄R expression following the induction of acute and chronic, inflammatory and neuropathic pain conditions. Peripheral administration of CFA induces localised inflammation and inflammatory pain. Similarly, chronic constriction injury (CCI) of the sciatic nerve reduced the paw withdrawal latency to mechanical stimulation, consistent with the induction of mechanical allodynia.

Quantification of the protein extracted from skin excised from the plantar hind surface during the acute phase of inflammation revealed significant increase in expression of the H₄R. This is consistent with H₄R expressing leukocytes such as neutrophils, eosinophils, dendritic and mast cells infiltrating the site of the acute phase of inflammation. Pre-treatment with JNJ 7777120 during inflammatory conditions has been shown to prevent signs of inflammation, cellular chemotaxis (Coruzzi et al., 2012, Coruzzi et al., 2007) and inflammatory pain (Hsieh et al., 2010a). Since the H₄R is functionally expressed on the sensory neurons (Chapter 3), there is a strong possibility that the anti-nociceptive effects of antagonism are mediated through action on sensory pain pathway.

Current literature depicts the H₄R as having a role in inflammatory pain conditions (Hsieh et al., 2010a), where antagonists such as JNJ 7777120 exhibit anti-hyperalgesic activity in models of inflammatory (Coruzzi et al., 2007, Coruzzi et al., 2012) and neuropathic pain (Hsieh et al., 2010a). The observed analgesic effect appears secondary to the anti-inflammatory activity induced by H₄R antagonism on immune cells through inhibition of chemotaxis (Ling et al., 2004) and cytokine production (Cowden et al., 2010b). In addition, receptor levels change with progression of the pathophysiological response, for example, inflammatory stimuli have been shown to up-regulate H₄R expression in monocytes (Dijkstra et al., 2007). However, the results obtained in Chapter 3 suggest a potential neuronal acting site for the H₄R antagonists. While the significance of the differential changes remains unclear, it would be interesting to demonstrate the potential changes in expression of the closely related H₃R. H₃Rs are inhibitory heteroreceptors that are functionally expressed on a subset of DRG neurons and may regulate the release of histamine and other neurotransmitters such as substance P from DRG neurons. Immunohistochemical analysis revealed that the H₃R is expressed on A δ sensory fibres of the superficial spinal cord. Based on the results obtained in Chapter 4 with the anti-hH₄R antibody, it may underestimate the observed changes in H₄R expression that occur in the rat CNS during pain states. This is because the antibody used was generated towards the human H₄R, which shares 69% with the rat H₄R (Liu et al., 2001) and therefore an antibody specific to the rat H₄R is required.

Further experiments are required to observe H₄R expression during the pathogenesis of CFA-induced inflammation, comparing animal behaviour to H₄R expression in the skin and spinal cord during the chronic phases of acute and neuropathic inflammatory pain. It would be interesting to observe potential differential expression of the H₄R in the spinal cord during progression of pathogenesis. In addition, molecular experiments are required to find out if there is a relationship between H₄R activation and release of neuropeptides such as substance P and CGRP from nociceptive afferent terminals during conditions of inflammatory pain. Since JNJ777120 has been shown to dose-dependently reverse

carrageenan induced thermal hyperalgesia (Hsieh et al., 2010a), it would be of interest to observe the relationship between activation of the neuronal H₄R and ion channels such as the capsaicin receptor TRPV1, which is involved in the development of thermal hyperalgesia (Caterina et al., 1997, Linley et al., 2010).

3) Antagonism of the central histamine H₄ receptor exacerbates EAE

Much of the current published research into the pre-clinical effect of H₄R antagonism has revealed anti-inflammatory effects, in rodent models of inflammation. In contrast, H₄R antagonism with JNJ 7777120 aggravated central inflammation in the EAE mouse model of multiple sclerosis, exemplified by a higher number of inflammatory infiltrates and a higher rate of demyelination in the lumbar spinal cord. The H₄ receptor appears to have a modulatory role within the CNS as H₄R KO mice develop more severe EAE despite having T effector cell responses that were equal to those of wild type mice. The H₄ receptor KO mice present with augmented neuroinflammation and increased BBB permeability compared with wild type mice, despite comparable T effector cell responses.

Although JNJ777120 has been proven to have functional antagonism on calcium mobilisation and chemotaxis in leukocytes through the inhibition of adenylyl cyclase activation and cAMP (Thurmond et al., 2004b), and inhibition of calcium mobility in DRG cells (Chapter 3), it may have agonist properties in the CNS through activation of a pathway that does not involve calcium mobilisation. JNJ 7777120 has been demonstrated to be an antagonist at the human H₄R and a partial agonist at the mouse H₄R. We cannot exclude the possibility that the absence of H₄ receptor signalling contributes to the increased disease severity, because of disturbed CNS signalling functions. On the other hand, JNJ 7777120 could be acting as an agonist via the recruitment of β -arrestin (Rosethorne and Charlton, 2011, Wall, 1978), therefore worsening the clinical state of the animals. It is possible that the agonism and antagonism of the centrally expressed H₄ receptor may have different effects compared to the peripherally expressed H₄ receptor. Further work is needed for elucidating the pathway activated during central H₄ receptor agonism and antagonism, if specific H₄ receptor ligands are to

be among the group of potential drugs to treat inflammatory or autoimmune diseases and neuropathic pain secondary to CNS inflammatory disorders.

4) Antagonism of the H₄ receptor has a central effect but no effect on anxiety or novel object recognition memory

The elevated platform is a novel validated paradigm used to assess a number of behaviours such as anxiety, mobility, learning and memory. Despite the confirmed expression in the rodent and human brain (Connolly et al., 2009), the role of the H₄R is still unclear due to the lack of research. In the elevated platform, the slopes provide an incentive for the animals as they can cross onto the slopes and get closer to the ground. Mice exposed to an elevated platform do try to escape; animals show preference for the areas adjacent to slopes which provide a potential route for escape by jumping onto the ground when the platform is not sufficiently raised. Antagonism of the H₄R with JNJ 7777120 did not facilitate crossing onto the slopes of the platform, in contrast to diazepam treated where Balb/c mice did cross frequently and spent more time on the slopes (Ennaceur et al., 2010a). On the other hand, a study by Galeotti *et al.* (2013) showed that the H₄R agonist VUF 8430 had apparent anxiolytic effects by increasing the time spent in the lit compartment of the light/dark box. This effect was attributed to H₄R activation because antagonist JNJ 10191584 antagonised the effects of VUF 8430. Our laboratory has provided evidence that this latter test is not an accurate measure of anxiety, but rather an indication of avoidance responses, which may account for the differences in results (reference). In the novel object recognition test (non-spatial working memory task), JNJ 7777120 did not facilitate discrimination between novel and familiar object. JNJ 7777120 had no effect on any test parameter of the novel object recognition test including the number of crossings and time spent in the different areas of the platform. This suggests that antagonising the H₄R has no anxiolytic or novel object recognition memory enhancing effects in Balb/c mice. These studies however did **provide preliminary evidence for central**

behavioural effects with regard to time spent in central part of the platform, which is difficult to interpret at this stage. In order to fully understand the role of the central H₄R in behaviour, future experiments are required to investigate effects on other forms of memory, including use of scopolamine-induced amnesias, spatial tasks and social interactions (the latter studied with newer tests currently under development in our laboratory).

Understanding the biology of histamine and its respective receptors is vital for the development of novel therapies. Drugs targeting the histamine H₁R and histamine H₂R have been available for many years for the treatment of allergies and gastric inflammation, respectively. Expectations for similarly clinically effective drugs are expected for both the H₃R and H₄R. The discovery of the H₄R on haematopoietic cells suggested its potential as a target for the treatment of inflammation and allergies. The data obtained from pre-clinical models of chronic inflammatory pain and pruritus, show that H₄R antagonists relieve these symptoms with greater efficacy than clinically prescribed H₁R antagonists.

The involvement of the H₄ receptor in the sensory pathway has been further supported by the expression of H₄R on peripheral afferent fibres. While the H₄ receptor clearly has potential as a therapeutic target, predicting the effect of any ligand is difficult when faced with the complexity observed within and between species. This thesis has furthered the understanding of the H₄ receptor expression and pharmacological profile within the peripheral and central nervous system. The experiments described will need to be extended to investigate the full range of receptor function and how they interact with other cellular components, whilst incorporating the use of H₄R^{-/-} animals. Whether alternative slicing in H₄ receptors has functional relevance in the CNS is yet to be seen. The functional expression of this G protein coupled receptor in the CNS will enable the characterisation of the H₄R involvement in neurological diseases particularly pain disorders and dermatitis where inflammation plays an active role.

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ABSTRACTS

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